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(54) Title: NOVEL TRANSGENE EXPRESSION SYSTEM FOR INCREASED PERSISTENCE

## (57) Abstract

The invention is directed to a transgene expression system comprising a transcription unit which contains a transgene operably linked to expression control sequences, preferably the CMV promoter, and which is delivered simultaneously with all or part of the adenovirus E4 genomic region to a cell in order to facilitate persistent expression of the transgene. The components of the transgene expression system can be delivered by vectors including plasmids and/or viruses and may be complexed with cationic amphiphiles to facilitate entry into a cell. The invention is also directed to methods for the production of the transgene expression system. The invention is further directed to compositions that contain the transgene expression system and to methods for the use of such compositions to deliver transgenes encoding biologically active proteins to cells.

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Description

Novel Transgene Expression System for Increased Persistence

5      Introduction

The invention is directed to a transgene expression system which contains a transcription unit containing a transgene operably linked to expression control sequences, preferably the cytomegalovirus immediate early promoter, and which is delivered concurrently with all or part of the adenovirus E4 genomic region to a cell in order to facilitate persistent expression of the transgene in the cell. The components of the transgene expression system can be delivered by one or more vectors, including plasmids and/or viruses and may be complexed with cationic amphiphiles to facilitate entry into a cell. The invention is also directed to methods for the production of the transgene expression system. The invention is further directed to compositions containing the transgene expression system and to methods for the use of such compositions to deliver transgenes encoding biologically active proteins to cells *in vivo* and obtain persistent expression thereof.

10     Background of the Invention

Adenovirus is a non-enveloped, nuclear DNA virus with a genome of about 36 kb, which has been well-characterized through studies in classical genetics and molecular biology (Horwitz, M.S., "Adenoviridae and Their Replication," in *Virology*, 2nd edition, Fields et al., eds., Raven Press, New York, 1990). The viral genes are classified into early (known as E1-E4) and late (known as L1-L5) transcriptional units, referring to the generation of two temporal classes of viral proteins. The demarcation between these events is viral DNA replication.

Recombinant adenoviruses have several advantages for use as gene transfer vectors, including tropism for both dividing and non-dividing cells, minimal pathogenic potential, ability to replicate to high titer for preparation of vector stocks, and the

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potential to carry large inserts (Berkner, K.L., Curr. Top. Micro. Immunol. 158:39-66, 1992; Jolly, D., Cancer Gene Therapy 1:51-64, 1994).

The cloning capacity of an adenovirus vector is proportional to the size of the adenovirus genome present in the vector. For example, a cloning capacity of about 8 kb can be created from the deletion of certain regions of the virus genome dispensable for virus growth, e.g., E3, and the deletion of a genomic region such as E1 whose function may be restored in trans from 293 cells (Graham, F.L., J. Gen. Virol. 36:59-72, 1977) or A549 cells (Imler et al., Gene Therapy 3:75-84, 1996). Such E1-deleted vectors are rendered replication-defective. The upper limit of vector DNA capacity for optimal carrying capacity is about 105%-108% of the length of the wild-type genome. Further adenovirus genomic modifications are possible in vector design using cell lines which supply other viral gene products in trans, e.g., complementation of E2a (Zhou et al., J. Virol. 70:7030-7038, 1996), complementation of E4 (Krougliak et al., Hum. Gene Ther. 6:1575-1586, 1995; Wang et al., Gene Ther. 2:775-783, 1995), or complementation of protein IX (Caravokyri et al., J. Virol. 69:6627-6633, 1995; Krougliak et al., Hum. Gene Ther. 6:1575-1586, 1995). Maximal carrying capacity can be achieved using adenoviral vectors deleted for all viral coding sequences (Kochanek et al., Proc. Natl. Acad. Sci. USA 93:5731-5736, 1996; Fisher et al., Virology 217:11-22, 1996).

Transgenes that have been expressed to date by adenoviral vectors include p53 (Wills et al., Human Gene Therapy 5:1079-188, 1994); dystrophin (Vincent et al., Nature Genetics 5:130-134, 1993; erythropoietin (Descamps et al., Human Gene Therapy 5:979-985, 1994; ornithine transcarbamylase (Stratford-Perricaudet et al., Human Gene Therapy 1:241-256, 1990; We et al., J. Biol. Chem. 271:3639-3646, 1996); adenosine deaminase (Mitani et al., Human Gene Therapy 5:941-948, 1994); interleukin-2 (Haddada et al., Human Gene Therapy 4:703-711, 1993); and  $\alpha$ 1-antitrypsin (Jaffe et al., Nature Genetics 1:372-378, 1992); thrombopoietin (Ohwada et al., Blood 88:778-784, 1996); and cytosine deaminase (Ohwada et al., Hum. Gene Ther. 7:1567-1576, 1996).

The particular tropism of adenoviruses for cells of the respiratory tract has particular relevance to the use of adenovirus in gene therapy for cystic fibrosis (CF),

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which is the most common autosomal recessive disease in Caucasians. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that disturb the cAMP-regulated Cl<sup>-</sup> channel in airway epithelia result in pulmonary dysfunction (Zabner et al., Nature Genetics 6:75-83, 1994). Adenovirus vectors engineered to carry the CFTR gene have been developed (Rich et al., Human Gene Therapy 4:461-476, 1993) and studies have shown the ability of these vectors to deliver CFTR to nasal epithelia of CF patients (Zabner et al., Cell 75:207-216, 1993), the airway epithelia of cotton rats and primates (Zabner et al., Nature Genetics 6:75-83, 1994), and the respiratory epithelium of CF patients (Crystal et al., Nature Genetics 8:42-51, 1994). Recent studies have shown that administering an adenoviral vector containing a DNA sequence encoding CFTR to airway epithelial cells of CF patients can restore a functioning chloride ion channel in the treated epithelial cells (Zabner et al., J. Clin. Invest. 97:1504-1511, 1996; allowed U.S. Patent Application Serial No. 08/136,742, filed October 13, 1993).

The use of adenovirus vectors in gene transfer studies to date indicates that persistence of transgene expression is often transient. At least some of the limitation is due to the generation of a cellular immune response to the viral proteins which are expressed antigenically even from a replication-defective vector, triggering a pathological inflammatory response which may destroy or adversely affect the adenovirus-infected cells (Yang et al., J. Virol. 69:2004-2015, 1995; Yang et al., Proc. Natl. Acad. Sci. USA 91:4407-4411, 1994; Zsengeller et al., Hum Gene Ther. 6:457-467, 1995; Worgall et al., Hum. Gene Ther. 8:37-44, 1997; Kaplan et al., Hum. Gene Ther. 8:45-56, 1997). Because adenovirus does not integrate into the cell genome, host immune responses that destroy virions or infected cells have the potential to limit adenovirus-based gene therapy. An adverse immune response poses a serious obstacle for high dose administration of an adenovirus vector or for repeated administration (Crystal, R., Science 270:404-410, 1995).

In order to circumvent the host immune response which limits the persistence of transgene expression, various strategies have been employed, generally involving either the modulation of the immune response itself or the engineering of a vector that decreases

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the immune response. The administration of immunosuppressive agents together with vector administration has been shown to prolong transgene persistence (Fang et al., Hum. Gene Ther. 6:1039-1044, 1995; Kay et al., Nature Genetics 11:191-197, 1995; Zsellenger et al., Hum. Gene Ther. 6:457-467, 1995).

5       The lack of persistence in the expression of adenovirus vector-delivered transgenes may also be due to limitations imposed by the choice of promoter or transgene contained in the transcription unit (Guo et al., Gene Therapy 3:802-801, 1996; Tripathy et al., Nature Med. 2:545-550, 1996).

10      Modifications to genomic adenoviral sequences contained in the recombinant vector have been attempted in order to decrease the host immune response (Yang et al., Nature Genetics 7:362-369, 1994; Lieber et al., J. Virol. 70:8944-8960, 1996; Gorziglia et al., J. Virol. 70:4173-4178; Kochanek et al., Proc. Natl. Acad. Sci. USA 93:5731-5736, 1996; Fisher et al., Virology 217:11-22, 1996). The adenovirus E3 gp19K protein can complex with MHC Class I antigens and retain them in the endoplasmic reticulum, which prevents cell surface presentation and killing of infected cells by cytotoxic T-lymphocytes (CTLs) (Wold et al., Trends Microbiol. 4:37-44, 1994), suggesting that its presence in a recombinant adenoviral vector may be beneficial. Experiments in which adenovirus vectors were introduced into nude mice demonstrated that the context of the adenovirus E4 genomic region was a determinant in the persistence of expression, especially when the CMV promoter was used to control expression of the transgene (Kaplan et al., Hum. Gene Ther. 8:45-56, 1997; Armentano et al., J. Virol. 71:2408-2416, 1997).

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Therefore, the current state of adenoviral vector-based gene delivery requires the development of transgene expression systems which demonstrate the capability for persistence and sustained expression of a transgene.

#### Summary of the Invention

The invention is directed to a transgene expression system which contains a transcription unit containing a transgene operably linked to expression control sequences,

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preferably the cytomegalovirus immediate early promoter, and which is delivered concurrently with all or part of the adenovirus E4 genomic region to a cell in order to facilitate persistent expression of the transgene. The components of the transgene expression system can be delivered by one or more plasmids and/or viruses and may be complexed with cationic amphiphiles to facilitate entry into a cell. The invention is also directed to methods for the production of the transgene expression system. The invention is further directed to compositions that contain the transgene expression system and to methods for the use of such compositions to deliver transgenes to cells *in vivo* and obtain persistent expression thereof.

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#### Brief Description of the Drawings

Figure 1 shows a schematic diagram of the genomic structure of the CMV $\beta$ gal, E1aCFTR and E1a $\beta$ gal adenoviral vector series.

Figure 2 shows a schematic diagram of the Ad2/ $\beta$ gal adenoviral vector series.

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Figure 3 shows a schematic diagram of the Ad2/CMVCFT adenoviral vector series.

Figure 4 shows the effect of adenovirus E4 on  $\beta$ -galactosidase expression in nude mice.

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Figure 5 shows the effect of adenovirus E4 on  $\beta$ -galactosidase expression in rat hepatocytes.

Figure 6 shows the effect of adenovirus E4 on  $\beta$ -galactosidase expression in rat hepatocytes.

Figure 7 shows the expression of  $\beta$ -galactosidase using E4-modified adenoviral vectors in nude mice.

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Figure 8 shows the expression of CFTR from E4-modified adenoviral vectors in mice.

Figure 9A shows the expression of  $\beta$ -galactosidase from E4-modified adenoviral vectors in rat hepatocytes. Figure 9B shows expression using adenoviral vectors deleted for specific E4 open reading frames.

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Figure 10 shows a schematic diagram of plasmid pCF1-CAT.

Figure 11 shows the persistence of expression of  $\beta$ -galactosidase from an adenoviral vector in nude mice.

5 Figure 12 shows the persistence of expression of CAT from pCF1-CAT in nude mice at 16 days.

Figure 13 shows the persistence of expression of CAT from pCF1-CAT in nude mice at 28 days.

Figure 14 shows the persistence of expression of CAT relative to promoter choice.

Figure 15 shows the persistence of expression of CAT in immunocompetent mice.

10 Figure 16 shows the persistence of expression of CAT in nude rats.

Figure 17 shows the reactivation of expression of CAT by the provision of E4+ adenovirus.

Figure 18 shows the persistence of expression of CAT following intravenous administration.

15 Figure 19 shows the persistence of expression of CAT expressed from a wild-type or truncated CMV promoter in nude mice.

Figure 20 shows a schematic diagram of a deletion series of the CMV promoter relative to persistent expression in the presence of the E4 gene.

20 Figure 21 shows the persistence of expression relative to promoter used in plasmid.

Figure 22 shows persistence of CAT expression in the presence of plasmid-encoded E4 sequences.

Figure 23 is a schematic diagram of pCFA-ORF3-CAT.

25 Figure 24 shows the persistence of expression of CAT in the presence of plasmid-encoded ORF 3 and adenovirus.

#### Detailed Description of the Invention

The invention is directed to a transgene expression system which comprises DNA sequences encoding a transcription unit containing a transgene operably linked to

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expression control sequences, preferably the cytomegalovirus (CMV) immediate early promoter, and DNA sequences encoding all or part of the adenovirus E4 genomic region, which are simultaneously delivered to a cell in order to facilitate persistent expression of the transgene. These components of the transgene expression system can be delivered by one or more plasmids and/or viruses and may be complexed with cationic amphiphiles to facilitate entry into a cell. The invention is also directed to methods for the production of the transgene expression system. The invention is further directed to compositions that contain the transgene expression system and to methods for the use of such compositions to deliver transgenes to cells in vivo and obtain persistent expression thereof.

Transgene is defined herein as any gene that is not native to the adenovirus genome. Persistent expression is defined as generating a sustained level of expression of a transgene over time.

The invention is also directed to novel adenoviral vectors which are capable of providing for persistent expression of the transgene because the vector contains all components of the transgene expression system of the invention. The vector contains a transgene under the control of the CMV promoter. The adenovirus E4 region is preferably retained in the vector to increase the persistence of expression from the transgene under the control of the CMV promoter. The adenovirus genome of the vector may contain other modifications to the adenovirus genome e.g., is deleted for all or part of the E1 region and all or part of the E3 region.

The transcription unit of the transgene expression system of the invention is defined herein as the DNA sequences encoding a transgene, any expression control sequences such as a promoter or enhancer, a polyadenylation element, and any other regulatory elements that may be used to modulate or increase expression, all of which are operably linked in order to allow expression of the transgene. The use of any expression control sequences, or regulatory elements, which facilitate persistent expression of the transgene is within the scope of the invention. Such sequences or elements may be capable of generating tissue-specific expression or be susceptible to induction by exogenous agents or stimuli.

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Preferably, the cytomegalovirus (CMV) immediate early promoter (Boshart et al., Cell 41:521-530, 1985) is used to control expression of the transgene in a transcription unit, or a truncated fragment of this promoter which functions analogously may be used.

The CMV promoter is positioned 5' to the transgene in a transcription unit. Portions of the full-length promoter can be tested for their ability to allow persistent expression of a transgene using assays described below. Preferably, the region of the CMV promoter from nucleotides -523 to -14 or -522 to +72 may be used in the transgene expression system of the invention. A further preferred embodiment for use in the invention is a CMV promoter comprising nucleotides -522 to +72, with a deletion from

-462 to -141. Other preferred embodiments of the CMV promoter include those having nucleotide sequences as follows: -140 to +72, -299 to -19, -140 to -19, or -19 to +72.

Other promoters can be used in a transcription unit of the invention, including, but not limited to, the Rous Sarcoma Virus (RSV) or human mucin 1 (MUC1) promoters.

Polyadenylation signals which may be positioned at the 3' end of the transgene in a transcription unit include, but are not limited to, those derived from bovine growth hormone (BGH) and SV40.

The use of an intron positioned into a transcription unit of the invention in order to activate the splicing apparatus and enhance message stability is within the scope of the invention. A preferred intron for such use is the hybrid intron described in Yew et al., Hum. Gene Ther. 8:575-584, 1997.

Transgenes which can be delivered and expressed from a transcription unit of the invention include, but are not limited to, those encoding enzymes, blood derivatives, hormones, lymphokines such as the interleukins and interferons, coagulants, growth factors, neurotransmitters, tumor suppressors, apolipoproteins, antigens, and antibodies, and other biologically active proteins. Specific transgenes which may be encoded by the transcription units of the invention include, but are not limited to, cystic fibrosis transmembrane regulator (CFTR), dystrophin, glucocerebrosidase, tumor necrosis factor, p53, retinoblastoma (Rb), and adenosine deaminase (ADA). Transgenes encoding antisense molecules or ribozymes are also within the scope of the invention.

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A further component of the transgene expression system of the invention is all or part of the adenovirus E4 genomic region which is provided concurrently with the transcription unit to a cell in order to prevent transcriptional down-regulation of transgene expression and therefore facilitate increased persistence. The gene products of the E4 region may be provided in trans to maintain persistence since the E4 DNA sequence is not required in cis to the transcription unit in order to function. The E4 region contains several open reading frames (ORF), including ORFs 1, 2, 3, 3/4, 6, and 6/7. The adenovirus E4 genomic region may be provided as a full length sequence, or portions of the E4 region or individual open reading frames may be used which function analogously to the full-length sequence to promote persistent expression of the transgene contained in the transcription unit. Where individual open reading frames of the E4 region are used to prevent transcriptional down-regulation of the transgene, such genes may be placed under the control of the native E4 promoters, or, alternatively, may be placed under the control of heterologous promoters. In one embodiment of the invention, the E4 open reading frames may be placed under the control of a promoter that causes sufficient expression of the E4 open reading frame(s) so that sufficient protein product is available for the transcription unit, e.g., using the CMV promoter separately or a promoter of similar strength.

In a preferred embodiment of the invention, the adenovirus E4 sequences provided on a plasmid or adenoviral vector retain the coding sequences for the ORF3 gene in order to facilitate persistent expression. In a particularly preferred embodiment of the invention, the adenoviral vector Ad2/CFTR-18 is used to provide persistent expression from the CFTR gene or the adenoviral vector backbone contained therein is used to provide persistent expression from another desired transgene.

It will be recognized by those skilled in the art that transient expression of a transgene, where desired, may be achieved using adenoviral vectors which are deleted for those components which confer persistent expression, e.g., removal of the E4 region or specific open reading frames contained therein.

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The components of the transgene expression system of the present invention may be delivered to a cell on one or more vectors, which include, but not limited to, plasmids and viruses. In a specific embodiment of the invention, one or more transcription units may be provided on a plasmid, where the CMV promoter is used to control expression and is positioned 5' to a transgene. As noted above, the adenovirus E4 component of the transgene expression system can be provided in cis or in trans to the transgene transcription unit. In a preferred embodiment of the invention, the adenovirus E4 region is provided in cis to the transgene transcription unit where the E4 region is engineered into the same vector, e.g., plasmid or virus, as the transcription unit. This particular embodiment is advantageous in that only one vehicle is required to deliver all DNA sequences required for persistent expression of the transgene. Alternatively, the E4 region may be provided in trans to the transcription unit on a separate vector that is co-delivered to a cell.

The components of the transgene expression system can be delivered to a cell using a hybrid vector delivery system in which some of the components are delivered by plasmid, and other components are delivered on a virus. Preferably, such a hybrid delivery system contains the transgene transcription unit on a plasmid, and contains the adenovirus E4 sequences in trans on a virus. In a preferred embodiment, the virus is an adenovirus. Delivery of the transgene expression system using this hybrid vector system also can utilize cationic molecule-based delivery, for example, to deliver both plasmid and virus, or, alternatively, may use cationic molecule-based delivery to deliver the plasmid to a cell and allow the virus to infect the cell simultaneously. In a preferred embodiment of the invention, both plasmid and virus are co-delivered by the same transfer route, e.g., cationic molecule-based delivery, in order to control the stoichiometry of the transgene expression system components. In one embodiment of the invention, the plasmid and virus need not be delivered simultaneously and can be sequentially delivered over, e.g., several days. For example, the plasmid may be delivered to the cell and reactivated by subsequent delivery of the virus at a later time. Reactivation of a delivered

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plasmid may also be accomplished by the administration of such molecules as sodium butyrate.

Novel adenovirus vectors can be used to deliver the components of the transgene expression system and are within the scope of the invention. In this embodiment of the invention, an adenoviral vector is engineered to contain the transgene transcription unit, wherein the transgene is preferably under the control of the CMV promoter, as well as retaining all or part of the E4 genomic region. The virus is preferably a replication-defective adenoviral vector, which may contain all or part of the other genomic regions of the adenovirus genome, e.g., E1, E2 and E3, and in which the transgene transcription unit is preferably inserted into a deleted E1 region. In a preferred embodiment of the invention, such an adenoviral vector retains the coding sequences for the E4 ORF3 gene and E3 gp19K gene. Examples of vector backbones which can be used to contain a transcription unit and which include the adenovirus E4 region include Ad2/CFTR-1 (U.S. Patent No. 5,670,488 issued September 23, 1997; Rich et al., Human Gene Therapy 4:461-476, 1993, incorporated herein by reference). It is within the level of the skilled artisan to construct adenoviral vectors which contain a transcription unit of the invention engineered into such vectors.

To create the recombinant adenoviral vectors of the invention which contain a transcription unit, a plasmid containing the transcription unit inserted into an adenovirus genomic fragment is co-transfected with a linearized viral genome derived from an adenoviral vector of interest into a recipient cell under conditions whereby homologous recombination occurs between the genomic fragment and the virus. Preferably, the transcription unit is engineered into the site of an E1 deletion. As a result, the transcription unit encoding a desired transgene is inserted into the adenoviral genome at the site in which it was cloned into the plasmid, creating a recombinant adenoviral vector. Following the homologous recombination, the vector genome is encapsidated into virions as evidenced by the formation of viral plaques. Preparation of replication-defective vector stocks which contain the transcription unit and/or the adenovirus E4 region can be accomplished using cell lines that complement viral genes deleted from the vector, e.g.,

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293 or A549 cells containing the deleted adenovirus E1 genomic sequences. After amplification of plaques in suitable complementing cell lines, the viruses can be recovered by freeze-thawing and subsequently purified using cesium chloride centrifugation. Alternatively, virus purification can be performed using chromatographic techniques, e.g., as set forth in International Application No. PCT/US96/13872, filed 5 August 30, 1996, incorporated herein by reference..

Titers of replication-defective adenoviral vector stocks can be determined by plaque formation in a complementing cell line, e.g., 293 cells. For example, end-point dilution using an antibody to the adenoviral hexon protein may be used to quantitate virus production (Armentano et al., Hum. Gene Ther. 6:1343-1353, 1995).

Plasmids which may be used to deliver the transgene transcription unit and/or the adenovirus E4 sequence include such vectors as pCMV $\beta$  (Clontech, Palo Alto, CA), in which a transgene can be placed under the control of the CMV promoter, or pCFA-15 ORF3-CAT. The invention contemplates the use of any plasmids into which the components of the transgene expression system can be engineered using standard recombinant DNA technology and which facilitate the persistent expression of the transgene.

Other vectors, such as plasmids containing the transcription unit and /or the adenovirus E4 region of the transgene expression system can be constructed using 20 standard techniques of recombinant DNA technology. Large scale production and purification can be performed using techniques well known to those skilled in the art (see, e.g., Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons, Inc., New York, 1995).

The DNA components of the transgene expression system are preferably 25 contained on various vectors, including plasmids and/or viruses for delivery to a cell. However, the delivery of such components to a cell in the form of naked DNA is also within the scope of the invention, using such routes as transfection, electroporation, microinjection, and other DNA transfer methods. Such transfer may be facilitated by the

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use of cationic molecules, such as cationic lipids as disclosed in PCT Publication No. WO96/18372, published June 20, 1996, incorporated herein by reference..

Where a marker or reporter gene is used as the transgene to determine the persistence of expression in the transgene expression system of the invention, a plasmid such as pCF1-CAT, containing the chloramphenicol acetyltransferase (CAT) gene under the control of the CMV promoter (described in Example 6, *infra*), can be used. Other marker genes which can be used include, but are not limited to, the genes encoding  $\beta$ -galactosidase and luciferase.

The transgene expression system can be used to facilitate persistence by preventing down-regulation of transgene expression from expression control sequences. In a particular embodiment of the invention, the transgene expression system components can be used in any vector or gene delivery system, *e.g.*, a retroviral vector containing a transgene operably linked to the CMV promoter and further containing all or part of the adenovirus E4 region.

Where any or all of the components of the transgene expression system are contained on one or more plasmids and/or viruses, the entry of such vehicles into a cell may be accomplished using mediated delivery, such as with the use of cationic amphiphiles, including lipids.

Cationic amphiphiles have a chemical structure which encompasses both polar and non-polar domains so that the molecule can simultaneously facilitate entry across a lipid membrane with its non-polar domain while its cationic polar domain attaches to a biologically useful molecule to be transported across the membrane.

Cationic amphiphiles which may be used to form complexes with the plasmids or viruses containing the transgene expression system of the invention include, but are not limited to, cationic lipids, such as DOTMA (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413-7417, 1987) (N-[1-(2,3-dioletloxy)propyl]-N,N,N - trimethylammonium chloride); DOGS (dioctadecylamidoglycylspermine) (Behr et al., Proc. Natl. Acad. Sci. USA 86:6982-6986, 1989); DMRIE (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide) (Felgner et al., J. Biol. Chem. 269:2550-2561, 1994; and DC-chol

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(3B [N-N', N'-dimethylaminoethane] -carbamoyl) cholesterol) (U.S. Patent No. 5,283,185 to Epand et al.). The use of other cationic amphiphiles recognized in the art or which come to be discovered is within the scope of the invention.

In preferred embodiments of the invention, the cationic amphiphiles useful to complex with and facilitate transfer of the plasmids and/or viruses of the invention are those lipids which are described in PCT Publication No. WO96/18372, published June 20, 1996, which is incorporated herein by reference. Preferred cationic amphiphiles described herein to be used in the delivery of the plasmids and/or viruses are GL-53, GL-67, GL-75, GL-87, GL-89, and GL-120, including protonated, partially protonated, and deprotonated forms thereof. Further embodiments include the use of non-T-shaped amphiphiles as described on pp. 22-23 of the aforementioned PCT application, including protonated, partially protonated and deprotonated forms thereof. Most preferably, the cationic amphiphile which is used to deliver the plasmids and/or viruses of the invention containing the components of the transgene expression system is spermine cholesterol carbamate (GL-67).

In the formulation of compositions comprising the transgene expression system of the invention, one or more cationic amphiphiles may be formulated with neutral co-lipids such as dileoylphosphatidylethanolamine (DOPE) to facilitate delivery of the transgene expression system into a cell. Other co-lipids which may be used in these complexes include, but are not limited to, diphytanoylphosphatidylethanolamine, lyso-phosphatidylethanolamines, other phosphatidylethanolamines, phosphatidylcholines, lyso-phosphatidylcholines and cholesterol. A preferred molar ratio of cationic amphiphile to colipid is 1:1. However, it is within the scope of the invention to vary this ratio, including also over a considerable range. In a preferred embodiment of the invention, the cationic amphiphile GL-67 and the neutral co-lipid DOPE are combined in a 1:2 molar ratio, respectively, before complexing with a plasmid and/or virus for delivery to a cell.

In one embodiment of the invention, where the transgene transcription unit is contained on a plasmid and the adenovirus E4 region is contained in an adenoviral vector,

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both plasmid and virus may be complexed with a cationic amphiphile for delivery to a cell. For example, the plasmid may be combined with GL-67:DOPE (1:2 molar ratio), and the adenovirus may be combined with 100% GL-67, and equal volumes of each of these may be combined to form the plasmid/virus complex containing all components of the transgene expression system.

In the formulation of complexes containing a cationic amphiphile with a plasmid, a preferred range of from .4 mM - 1 mM of cationic amphiphile may be combined with a range of 3 mM - 8 mM of plasmid to form the complexes. In the formulation of complexes containing a cationic amphiphile with a virus, preferably adenovirus, a preferred range of  $10^7$  -  $10^{10}$  infectious units of virus may be combined with a range of  $10^4$  -  $10^6$  cationic amphiphile molecules/viral particle.

Assays may be performed in tissue culture systems to determine the persistence of expression of a transgene *in vivo*. Cell lines which may be transfected with the plasmids or infected with the viruses of the invention are suitable for assays which measure the level and duration of expression of a contained transgene. The transgene may encode a biologically useful protein or may encode a marker protein used to test persistence of the transgene expression system *in vivo*. Relevant molecular assays to determine the persistence of expression include the measurement of transgene mRNA, by, for example, Northern blot, S1 analysis or reverse transcription-polymerase chain reaction (RT-PCR). The presence of a protein encoded by a transgene may be detected by Western blot, immunoprecipitation, immunocytochemistry, or other techniques known to those skilled in the art.

In order to determine the persistence of transgene expression *in vivo* using the constructs and compositions of the invention, animal models are particularly relevant in order to assess transgene persistence against a background of potential host immune response. Such a model may be chosen with reference to such parameters as ease of delivery, identity of transgene, relevant molecular assays, and assessment of clinical status. Where the transgene encodes a protein whose lack is associated with a particular

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disease state, an animal model which is representative of the disease state may optimally be used in order to assess a specific phenotypic result and clinical improvement.

Relevant animals in which the transgene expression system may be assayed include, but are not limited to, mice, rats, monkeys, and rabbits. Suitable mouse strains in which the transgene expression system may be tested include, but are not limited to, C3H, C57Bl/6 (wild-type and nude) and Balb/c (available from Taconic Farms, Germantown, New York).

Where it is desirable to assess the host immune response to vector administration, testing in immune-competent and immune-deficient animals may be compared in order to define specific adverse responses generated by the immune system. The use of immune-deficient animals, e.g., nude mice or rats, may be used to characterize vector performance and persistence of transgene expression, independent of an acquired host response, and to identify other determinants of transgene persistence.

In a particular embodiment where the transgene is the gene encoding cystic fibrosis transmembrane regulator protein (CFTR) which is administered to the respiratory epithelium of test animals, persistence of expression of CFTR may be assayed in the lungs of relevant animal models, for example, C57Bl/6 or Balb/c mice, cotton rats, or Rhesus monkeys. Molecular markers which may be used to determine the persistence of expression include the measurement of CFTR mRNA, by, for example, Northern blot, S1 analysis or RT-PCR. The presence of the CFTR protein may be detected by Western blot, immunoprecipitation, immunocytochemistry, or other techniques known to those skilled in the art. Such assays may also be used in tissue culture where cells deficient in a functional CFTR protein and into which the transgene expression system has been introduced may be assessed to determine the presence of functional chloride ion channels - indicative of the presence of a functional CFTR molecule.

The transgene expression system of the invention can be used to deliver and express any number of transgenes to cells in order to achieve a particular phenotypic result.

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The present invention is further directed to compositions containing the transgene expression system of the invention which can be administered in an amount effective to deliver one or more desired transgenes to the cells of an individual in need of such molecules and cause persistent expression of a transgene encoding a biologically active protein to achieve a specific phenotypic result. The cationic amphiphile-plasmid complexes or cationic amphiphile-virus complexes may be formulated into compositions for administration to an individual in need of the delivery of the transgenes.

The compositions can include physiologically acceptable carriers, including any relevant solvents. As used herein, "physiologically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the compositions is contemplated.

Routes of administration for the compositions containing the transgene expression system include conventional and physiologically acceptable routes such as direct delivery to the target organ or tissue, intranasal, intravenous, intramuscular, subcutaneous, intradermal, oral and other parenteral routes of administration.

The invention is further directed to methods for using the compositions of the invention in vivo or ex vivo applications in which it is desirable to deliver one or more transgenes into cells such that the transgene produces a normal biological or phenotypic effect. In vivo applications involve the direct administration of the transgene expression system formulated into a composition to the cells of an individual. Ex vivo applications involve the transfer of the transgene expression system directly to autologous cells which are maintained in vitro, followed by readministration of the transduced cells to a recipient.

Dosage of the transgene expression system to be administered to an individual for persistent expression of a transgene encoding a biologically active protein and to achieve a specific phenotypic result is determined with reference to various parameters, including the condition to be treated, the age, weight and clinical status of the individual, and the

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particular molecular defect requiring the provision of a biologically active protein. The dosage is preferably chosen so that administration causes a specific phenotypic result, as measured by molecular assays or clinical markers. For example, determination of the persistence of a transgene expression system containing the CFTR transgene which is administered to an individual can be performed by molecular assays including the measurement of CFTR mRNA, by, for example, Northern blot, S1 or RT-PCR analysis or the measurement of the CFTR protein as detected by Western blot, immunoprecipitation, immunocytochemistry, or other techniques known to those skilled in the art. Relevant clinical studies which could be used to assess phenotypic results from delivery of the CFTR transgene include PFT assessment of lung function and radiological evaluation of the lung. Demonstration of the delivery of a transgene encoding CFTR can also be demonstrated by detecting the presence of a functional chloride channel in cells of an individual with cystic fibrosis to whom the vector containing the transgene has been administered (Zabner et al., J. Clin. Invest. 97:1504-1511, 1996). The persistence of transgene expression in other disease states can be assayed analogously, using the specific clinical parameters most relevant to the condition.

Dosages of an adenoviral vector which contains all the components of the transgene expression system and which are effective to provide persistent expression of a transgene encoding a biologically active protein and achieve a specific phenotypic result range from approximately  $10^8$  infectious units (I.U.) to  $10^{11}$  I.U. for humans.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated, each unit containing a predetermined quantity of active ingredient calculated to produce the specific phenotypic effect in association with the required physiologically acceptable carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly depend on the unique characteristics of the transgene expression system and the limitations inherent in the art of compounding. The principal active ingredient (the transgene expression system) is compounded for convenient and effective

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administration in effective amounts with the physiologically acceptable carrier in dosage unit form as discussed above.

Maximum benefit and achievement of a specific phenotypic result from administration of the transgene expression system of the invention may require repeated administration. Where an adenoviral vector is used to deliver some or all of the components of the transgene expression system, such repeated administration may involve the use of the same adenoviral vector, or, alternatively, may involve the use of different vectors which are rotated in order to alter viral antigen expression and decrease host immune response.

The practice of the invention employs, unless otherwise indicated, conventional techniques of protein chemistry, molecular virology, microbiology, recombinant DNA technology, and pharmacology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Current Protocols in Molecular Biology, Ausubel et al., eds., John Wiley & Sons, Inc., New York, 1995, and Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, PA, 1985.

The invention is further illustrated by the following specific examples which are not intended in any way to limit the scope of the invention.

#### **Example 1: Construction of Adenoviral Vectors**

##### **Methods**

Schematic diagrams of recombinant adenoviruses used in the experiments below illustrating specific transcription units and particular modifications to the adenovirus genome, including those to the E4 region, are depicted in Figure 1.

Ad2/βgal expression vectors. The CMVβgal expression cassette was constructed in a pBR322-based plasmid that contained Ad2 nucleotides 1-10,680 from which nucleotides 357-3328 were deleted. The deleted sequences were replaced with a cytomegalovirus immediate early promoter (nucleotides -523 to -14, obtained from pRC/CMV, Invitrogen, Carlsbad, CA), lacZ gene encoding β-galactosidase with a nuclear localization signal (Kalderon et al., Cell 39:499-509, 1984) and an SV40 polyadenylation

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signal (nucleotides 2533-2729, Liebowitz et al., Curr. Top. Micro. Immunol. 87:43-172, 1979).

The E1aCFTR and E1a $\beta$ gal expression cassettes were similarly constructed except the Ad2 deletion started from nucleotide 545. The cDNA for CFTR represents nucleotides 123-4622 of the published sequence and the lacZ gene was obtained as a NotI fragment from pCMV $\beta$  (Clontech, Palo Alto, CA).

The variations of the E4 region have been assigned a number, depending on the particular vector series. As shown in Figure 1, for the CMV  $\beta$ gal, E1a CFTR, and E1a $\beta$ gal vector series, a wild type (wt) E4 region is assigned the number 1 and the Ad2E40RF6 (Armentano et al., Human Gene Therapy 6:1343-1353, 1995, incorporated herein by reference) backbone is assigned the number 2. Variation number 3 (mORF6) is identical to 2 but includes an SV40 polyadenylation signal (nucleotides 2533-2729) inserted between the fiber region and ORF6. This modification corrects the reduced fiber synthesis that is observed with Ad2E4ORF6 (unpublished results). Variation number 4 (protein IX moved, PNM) is based on mORF6 and contains pIX sequences (nucleotides 3519-4061) that have been deleted and relocated between fiber and the SV40 polyadenylation signal. This modification helps to reduce the generation of replication competent adenovirus during virus expansion and has been invaluable for generating clinical grade preparations of virus (Hehir et al., J. Virol. 70:8459-8467, 1996). A complete E4 deletion, variation 5 ( $\Delta$ E4), was constructed as follows. pAdORF6 (Armentano et al., Hum. Gene Ther. 6:1344-1353, 1995) was cut with BamHI and Sall which removes the ITR and E4ORF6. This was replaced with a BamHI-BgIII fragment containing the SV40 polyadenylation signal and a BamHI-Sall fragment generated by PCR containing Ad nucleotides 35642-35937 (E4 enhancer region and inverted terminal repeat).

A schematic diagram of the Ad2/ $\beta$ gal vector series is shown in Figure 2. Ad2/ $\beta$ gal-7 was made in VK2-20 cells by recombination of the large PacI fragment of Ad2/ $\beta$ gal-2 (corresponding to the left end of the virus) with the large PmeI fragment of dl366+ORF4 (corresponding to the right end). Ad2/ $\beta$ gal-8 through -13 were similarly

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made in 293 cells using Ad2/βgal-5 for recombination. Ad2/βgal-8 was made by recombination with E4dlORF1-4 which contains only ORFs 6 and 6/7 (Huang et al., J. Virol. 63:2605-2615, 1989). Viruses with knockouts of individual ORFs Ad2/βgal-9 (ORF1), Ad2/βgal-10 (ORF2), Ad2/βgal-11 (ORF3), Ad2/βgal-12 (ORF4) and Ad2/βgal-13 (ORF6/7) were derived from in 351, in 352, E4inORF3, dl358, and dl356 respectively (Halbert et al., J. Virol. 56:250-257, 1985).

Some previously described vectors have been assigned a different name for simplicity. The vectors CMVβgal-1 and -2 refer to Ad2/βgal-2 and Ad2/βgal-4, respectively (Kaplan et al., Hum. Gene Ther. 8:45-56, 1997) and the vector E1aCFTR-4 (PNM) refers to Ad2/CFTR-8 (Hehir et al., J. Virol. 70:8459-8467, 1996).

Ad2/CFTR expression vectors. The construction of Ad2/CFTR-5 has been described (Jiang et al., Hum. Gene Ther. 8:671-680, 1997) and is modified in E4 to contain only ORF6. Ad2/CFTR-16 contains a wild-type E4 region but is modified in E3. Ad2/CFTR-18 is modified in E4 to contain only ORF3 and ORF4 and was constructed as follows. A plasmid containing an E4 ORF3 and ORF4 expression cassette was derived from pAdE4 which contains Ad2 sequences from 27123 to 35937 cloned into the SpeI and HindIII sites of pBluescriptIIsk+ (Stratagene). Sequences between the AvrII site (nucleotide 35470) and the BclI site (nucleotide 32891) in pAdE4 were deleted and replaced with a PCR generated AvrII-BamHI fragment (Ad2 nucleotides 34804 to 33998). This fragment contains the coding sequences for ORF3 and ORF4 as well as 3' splice sites for ORF3 and ORF4 expression. The resulting plasmid, pAdE4ORF3+4 was then linearized with EagI for recombination with PacI cut Ad2/CFTR-5 to generate Ad2/CFTR-18. These vectors are shown in Figure 3.

All viruses, except those with complete E4 deletions, were propagated in 293 cells, purified and titered by end-point dilution using FITC conjugated anti-hexon antibody (Chemicon) as previously described (Armentano et al., Hum. Gene Ther. 6:1344-1353, 1995). Viruses with complete E4 deletions were propagated in VK2-20 cells, which is an E4 complementing cell line derived from 293 cells (Krougliak et al.,

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Hum. Gene Ther. 6:1575-1586, 1995). Both 293 and VK2-20 cells were obtained from Dr. Frank Graham.

5       **Example 2: Persistence of Transgene Expression System Using Adenovirus E4 in  
Trans**

Methods

Experiments were performed to test whether the effect of E4 could be supplied in trans from a vector that contains a wild type E4 region but does not contain a CMV promoter. For this purpose, nude mice were infected with CMV $\beta$ gal-1 (wt E4) or CMV $\beta$ gal-2 (ORF6) alone or were co-infected with E1aCFTR-1 (wt E4), E1aCFTR-4 (PNM) or E1aCFTR-5 ( $\Delta$ E4). If a wild type E4 is required for persistence then co-infection of CMV $\beta$ gal-1 (wt E4) with any of the E1aCFTR series should not alter the expression profile and persistence of expression should be observed in all cases. If E4 is required but its effect can be supplied in trans then co-infection of CMV $\beta$ gal-2 (ORF6) with E1aCFTR-1 (wt E4) should result in persistence of expression similar to that which is observed with CMV $\beta$ gal-1 (wt E4). In contrast, co-infection with either E1aCFTR-4 (PNM) or -5 ( $\Delta$ E4) should not effect expression and an expression profile similar to that observed in animals that received CMV $\beta$ gal-2 alone would be expected.

20       Balb/c parental and nude mice were purchased from Taconic Farms (Germantown, New York). Animals, mostly females, ranging from 7 to 16 weeks old were used for in vivo studies. Mice were anesthetized by inhalation of Metofane (methoxyflurane) and were instilled intranasally with recombinant virus in 100 $\mu$ l PBS, 3% sucrose.

25       Mice were intranasally instilled with  $1.5 \times 10^9$  I.U. of CMV $\beta$ gal-1 (A-D) or CMV $\beta$ gal-2 (E-H) alone (A, E) or along with  $1.5 \times 10^9$  I.U. E1aCFTR-1 (B, F),  $1.5 \times 10^9$  I.U. E1aCFTR-4 (C, G) or  $4.3 \times 10^8$  I.U. E1aCFTR-5 (D, H). Mice were sacrificed on days 3 and 14, lungs from individual animals were homogenized, and  $\beta$ -galactosidase activity was measured by an AMPGD (3-(4-methoxyspiro [1,2-dioxethane-3,2'-tricyclo-

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[3.3.1.1<sup>3,7</sup>] decan]-4-yl) phenyl-β-D-galactopyranoside) (Galactolight™) assay (Tropix, Bedford, MA). The protein concentration in lung homogenates was measured with the BioRad (Hercules, CA) DC reagent and β-galactosidase activity is expressed as relative light units (RLU/μg protein). Each bar represents the average activity from at least three animals.

### Results

The results shown in Figure 4 indicate that the expression profile from CMVβgal-1 (A) remains unchanged in animals that were co-infected with any of the E1a-CFTR vectors (B, C and D). Expression from CMVβgal-2 (E) drops by day 14 in animals that were co-infected with E1aCFTR-4 or -5 (G,H). However, expression on day 14 in animals that were co-infected with E1aCFTR-1 (F) remains elevated and is similar to that seen in animals that received CMVβgal-1 (A-D). This suggests that an E4 product(s) is supplied in trans and can act, either directly or indirectly, to allow persistence of expression from the CMV promoter.

### **Example 3: Persistence of Transgene Expression System in Tissue Culture**

#### Methods

A co-infection study was performed in cultured rat hepatocytes to determine if an E4 requirement for expression from the CMV promoter would be observed in tissues other than the lung, and if the E4 function could be provided in trans. Primary rat hepatocytes were infected with CMVβgal-1 or -2 alone at an MOI (multiplicity of infection) of 10 or were co-infected at an MOI of 10 with E1aCFTR-1, -4 or -5 and were analyzed for β-galactosidase expression by X-gal staining.

#### Results

Figure 5, panel A and B, depict β-galactosidase expression observed with CMVβgal-1 on days 3 and 14 post-infection, respectively. Panels C, D and E correspond

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to expression observed 14 days post-infection in cultures that were co-infected with E1aCFTR-4, -1 and -5, respectively. In all cases, expression seems to persist to day 14.

Panels A and B in Figure 6 represent CMV $\beta$ gal-2 infected cultures analyzed on days 3 and 14 post-infection, respectively. Expression with this vector in hepatocytes has clearly diminished during this time period. Co-infection with E1aCFTR-4, -1 and -5, shown in panels C, D and E indicates that expression declines by day 14 except in the culture that was co-infected with E1aCFTR-1. These results demonstrate a requirement for E4 for prolonged expression from the CMV promoter in cultured hepatocytes as well as was the case in the mouse lung, and that the E4 function required for persistent expression can be provided in trans.

10

**Example 4: Effect of Specific E4 Open Reading Frames on The Persistence of Expression in The Mouse Lung**

**Methods**

15

Experiments were performed to determine whether specific open reading frames of the adenovirus E4 region were required for persistent expression of a transgene from the CMV promoter. Recombinant adenoviral vectors in which particular open reading frames of the E4 region were retained or deleted were used in the experiments.

20

Ad2/ $\beta$ gal expression vectors. Balb/c nude mice were intranasally instilled with  $3 \times 10^9$  infectious units of Ad2/ $\beta$ gal-4 (wild-type E4), Ad2/ $\beta$ gal-7 (containing ORF4), or Ad2/ $\beta$ gal-8 (containing ORF6 and ORF6/7) (Figure 2). Mice were sacrificed on days 3 and 14 and  $\beta$ -galactosidase activity in the lungs of the test animals was determined as described in Example 2, infra. Each time point represents the average from four animals.

25

Ad2/CFTR expression vectors. Mice were intranasally installed with  $3 \times 10^9$  infectious units of Ad2/CFTR-5, Ad2/CFTR-16 or Ad2/CFTR-18 and were sacrificed on days 3, 21 and 42 post-installation. RNA samples from lungs were pooled at each time point (n=4) with each vector and hCFTR mRNA was measured by quantitative RT-PCR.

**Results**

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Ad2/βgal expression vectors. Figure 7 shows that expression persisted to day 14 in animals that received Ad2/βgal-4, containing wild-type E4. Expression declined from day 3 to day 14 in animals that received Ad2/βgal-7 or Ad2/βgal-8. This suggests that neither ORF4 nor ORFs 6 and 6/7 are sufficient to achieve longevity of expression from the CMV promoter.

5

Ad2/CFTR expression vectors. The results shown in Figure 8 indicate that expression of CFTR persisted to day 42 in animals that received Ad2/CFTR-16 (wild-type E4) but not in animals that received Ad2/CFTR-18 (ORF3 and ORF4). This result supports the conclusion from expression analysis with the Ad2/Bgal vector series that ORF3 is required for long-term expression from the CMV promoter. In the Ad2/CFTR-10 18 vector, contributions on longevity of expression due to ORF4 cannot be ruled out. Since expression from the CMV promoter in a vector that retains only ORF4 (Ad2/βgal-7) does not persist, the contribution of ORF4 may be minimal. It is therefore most likely that ORF3 is sufficient for achieving long-term expression from the CMV promoter.

10

#### **Example 5: Effect of Specific E4 Open Reading Frames on The Persistence of Expression in Rat Hepatocytes**

##### Methods

Rat hepatocytes were infected with viruses in the Ad2/βgal series (Figure 2) at a MOI of 10. B-galactosidase expression was visualized by X-gal staining of the infected hepatocytes on days 3 and 14 post-infection.

15

##### Results

The graph in Figure 9A represents the percentage of stained cells in the cells infected with adenoviral vectors containing specific E4 open reading frames: Ad2/βgal-2, Ad2/βgal-7, and Ad2/βgal-8 (Figure 2). As previously observed, expression persists in rat hepatocytes with a vector that contains a wild-type E4 region (Ad2/βgal-4), but does not persist with a vector containing only ORF6 (Ad2/βgal-2). As shown here in the upper panel, expression does not persist in hepatocytes that were infected with either a

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vector containing only ORF 4 (Ad2/βgal-7) or a vector containing only ORFs 6 and 6/7 (Ad2/βgal-8). The data suggest that ORF4 or ORFs 6 and 6/7 are not sufficient to allow persistent expression from the CMV promoter.

The graph in Figure 9B represents results obtained in rat hepatocytes infected with adenoviral vectors containing knockouts of individual E4 ORFs (Ad2/βgal-9 through Ad2/βgal-13, Figure 2). Expression persists in all cultures infected with viruses containing individual ORF knockouts except the culture that was infected with the ORF3 knockout (Ad2/βgal-11). The data suggest that ORFs 1, 2, 4, and 6/7 are not required to achieve long-term expression and implicate ORF3 as playing a role in the observed effect of E4 on persistent expression from the CMV promoter. The data indicate that ORF3 is, at least, required to achieve persistence of expression from the CMV promoter.

**Example 6: Persistence of Hybrid Plasmid/Virus Transgene Expression System in Mice**

**15 Methods**

To create plasmid pCF1-CAT (Figure 10), the vector pCMVβ (Clontech, Palo Alto, CA) was first digested with NotI to excise the β-galactosidase gene to form pCMV containing the CMV immediate early promoter from -522 to +72 (Boshart et al., Cell 41:521-530, 1985). The bovine growth hormone (BGH) poly A signal was amplified from the vector pcDNA3 (Invitrogen, San Diego, CA) using the polymerase chain reaction (PCR). The 241 bp PCR product was subcloned into the vector pCRII (Invitrogen, San Diego) to form pCRII-BGH. The NotI-HindIII BGH fragment was excised and ligated into the NotI and HindIII sites of pCMV, replacing the SV40 poly A signal, to form pCMV-BGH. The hybrid intron was obtained from the vector pADB (Clontech, Palo Alto, CA). pADB was digested with PmlI and NotI, and the -500 bp fragment was isolated then ligated into the HincII and NotI sites of pBluescriptII KS(-) (Stratagene, La Jolla, CA) to form pBlueII-HI2. pBlueII-HI2 was digested with XhoI and NotI to excise the hybrid intron fragment. This fragment was ligated into the XhoI and NotI sites of pCMV-BGH, replacing the SV40 intron to form pCMVHI2BGH. The

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aminoglycoside 3'phosphotransferase (APT) gene, which confers resistance to kanamycin, was obtained from the vector pUC4K (Pharmacia, Piscataway, NJ). pUC4K was digested with EcoRI to excise the 1.3 kb APT gene. The EcoRI ends were blunted using the Klenow fragment of DNA polymerase I. pCMV was digested with AlwNI and 5 EcoRI and the ends blunted with Klenow. The APT fragment was ligated into the pCMV vector, replacing the ampicillin resistance gene to form pCMV/kan. pCMV/kan was digested with NcoI and AlwNI to excise the APT gene and flanking sequences. This fragment was ligated into the NcoI and AlwNI sites of pCMVHI2BGH to form 10 pCMVM2BGH/kan. The chloramphenicol acetyltransferase (CAT) cDNA was obtained from the CAT Geneblock (Pharmacia), and NotI linkers were added to the ends of the cDNA. pCMVHI2BGH/kan was digested with NotI and ligated to the CAT cDNA to 15 form pCF1-CAT (Figure 10).

The p5GΔCECMV-CAT (p5GminCMV-CAT) plasmid was derived from pCF1-CAT. A small polylinker was inserted 5' to the CMV enhancer/promoter of pCMVHI2BGH/kan (see above) to form pCMV(A-N)kan. Five yeast GAL4 binding sites were assembled from four synthetic oligonucleotides and inserted into the polylinker AvrII site of pCMV(A-N)kan. A 322 bp AatII fragment (deleting the CMV enhancer from -462 to -141 relative to the transcription start site) was removed, then the CAT cDNA was ligated into the NotI site to form p5GΔCE-CAT (p5GminCMV-CAT).

20 Cationic amphiphile-plasmid complexes were formed between GL67-DOPE (1:2) with pCF1-CAT (0.6:3.6 mM) and GL-67 (100%) with adenovirus (Ad2/CMV $\beta$ gal-4, containing wild type E4 or Ad2/CMV $\beta$ gal-5, lacking E4) (approximately  $3 \times 10^5$  GL-67 molecules/virus particle and about  $10^9$  infectious units/mouse). The lipid-DNA and lipid-virus complexes were formed by warming all components for 5 minutes to 30°C, 25 adding lipid to DNA or to virus with no agitation (140  $\mu$ l of lipid was added to 140  $\mu$ l of either DNA or virus), and allowing complexes to stand at 30° for 15 minutes. The two complexes were combined.

The mixture of both complexes was instilled intranasally into nude Balb/c mice in two doses of 50  $\mu$ l each. The animals were sacrificed at the time points indicated.

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Extracts from the lungs of the mice installed with the complexes were evaluated for  $\beta$ -gal activity generated from the adenovirus (positive control) and for CAT activity generated from the plasmid.

5       Lungs from individual animals were homogenized and  $\beta$ -galactosidase activity was assayed as described in Example 2, *infra*. CAT activity was detected by a standard isotopic assay (see, *e.g.*, short protocols in Molecular Biology, 3rd ed., Ausubel et al., eds., Wiley and Sons, New York, 1995).

### Results

10      The experiments were performed to determine if expression of the transgene could be achieved using a plasmid containing the transcription unit with a CMV promoter and an adenovirus containing the E4 region which were simultaneously introduced to the cell by cationic amphiphile-mediated delivery. The assays were performed at various times post-installation and the results are shown in Figures 11, 12, and 13. Transgene expression is shown as a function of the initial level of expression.

15      In Figure 11, the  $\beta$ -gal expression from the adenovirus demonstrated that the vector containing wild-type E4 (Ad2/CMV $\beta$ gal-4) showed persistence of expression over 16 days, with increasing levels of  $\beta$ -gal detectable over time. The vector deleted for E4 (Ad2/CMV $\beta$ gal-5) showed a rapid loss of  $\beta$ -gal expression (starting off from an absolute level about 50% of that from the Ad2/CMV $\beta$ gal-4 vector).

20      Figure 12 shows that the CAT activity from the plasmid vectors showed similar trends when instilled +/- wild-type E4 up to 6 days. The expression of CAT in the E4-deleted mice continued to decline over 16 days whereas the E4+ mice showed a steady increase up to about 70% of day 1 levels of expression (Figure 12). When CAT expression from pCF1-CAT was determined over a 28-day period, the presence of the adenovirus E4 region supplied by Ad2/ $\beta$ gal-4 correlated with significantly higher expression at day 28 than that detected in its absence (Figure 13). The results demonstrate the necessity of the E4 region for persistence of the plasmid-derived reporter gene expression.

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Identification of other promoters responsive to E4: Bolus intranasal instillations of complex to BALB/c nu/nu mice. The plasmids containing CAT driven from either a Rous Sarcoma Virus (RSV) promoter or a human mucin 1 (MUC1) promoter both gave persistent transgene expression in the presence of E4(+) adenovirus (Figure 14).

5 Persistent expression in immunocompetent animals: By co-instilling BALB/c mice with the less immunogenic reporter gene human  $\alpha$ -galactosidase and E4(+) adenovirus again persistent expression of  $\alpha$ -gal could be observed (Figure 15).

10 Persistent expression in an alternative species: Bolus intranasal instillations of complex to nude rats. In nude rats, as in nude mice, intranasal instillations for pCF1-CAT with E4(+) adenovirus resulted in more persistent transgene expression than with the E4(-) adenovirus (Figure 16).

15 Reactivation of plasmid by E4(+) adenovirus: Bolus intranasal instillations of pCF1-CAT:cationic lipid complex to BALB/c nu/nu mice with E4(+) adenovirus either co-instilled or instilled 7 days later resulted in a similar persistent expression profile. This indicates that the plasmid is still present in the lung cells and can be reactivated at a later timepoint (Figure 17).

20 Persistent expression following intravenous administration: Bolus intravenous injections of pCF1-CAT or pCFA-E4-CAT :cationic lipid complex to BALB/c nu/nu mice. Persistent expression was observed in lungs from the vector containing the E4 gene but not in the vector lacking E4 sequences (Figure 18).

**Example 7: Effect of Promoter Truncations on the Persistence of Expression from the CMV Promoter**

**Methods**

25 An experiment was performed to determine if the full length CMV promoter was required for persistent expression by the transgene expression system. Plasmids pCF1-CAT and p5GminCMV-CAT were introduced into nude Balb/c mice as described in Example 6, infra., together with the adenovirus E4 region supplied by Ad2/ $\beta$ gal-4, and expression was monitored for 14 days post-instillation.

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### Results

Figure 19 shows that persistence of CAT expression over 14 days is maintained in nude mice using the full pCF1-CAT promoter region or a plasmid containing a large deletion of part of the CMV promoter sequence (p5GminCMV-CAT).

5       Figure 20 shows a schematic diagram of a deletion series of the CMV promoter and the expression achieved with each promoter in response to the E4 gene.

### **Example 8: Persistence of CAT Expression from Different Promoter Plasmids in the Presence of an E4 Containing Adenovirus**

10       pCF1-CAT contains the CMV promoter, pRSV-CAT contains the Rous Sarcoma Virus LTR promoter, pCEMUC1-CAT contains the CMV enhancer-mucin 1 promoter, and pIL8-CAT contains the human interleukin-8 promoter. Nude BALB/c mice were co-instilled with Ad2/βgal-4 (E4+) and the different plasmids, then CAT assays were performed on lungs harvested at different days post-instillation.

15       The level of expression from plasmid vectors containing either the CMV, RSV, or CMV enhancer-mucin 1 promoters was approximately 80-300% of day 2 levels at day 21 when co-instilled with Ad2/βgal-4. In contrast, expression from these vectors was less than 5% of day 2 levels at day 21 without Ad2/βgal-4. Expression from the IL-8 promoter was not persistent in the presence of Ad2/βgal-4, with less than 5% of day 2 levels at day 21. This suggests that the CMV, RSV and CMV enhancer-mucin 1 20       promoters are responsive to E4, but the IL-8 promoter is not (Figure 21).

### **Example 9: Persistence of CAT Expression in the Persistence of Plasmid-Encoded E4 Sequences**

25       Nude BALB/c mice were co-instilled with a complex of lipid GL-67, pCF1-CAT and either pCFA-E4 or pCFA-ORF3, or a complex of cationic lipid GL-67 and pCFA-E4-CAT. CAT assays were performed on lungs harvested at different days post-instillation.

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As seen in Figure 22, the levels of CAT expression from mice that received plasmids that express either all of E4 or just ORF3 alone were significantly higher at day 42 compared to expression from mice instilled with pCF1-CAT alone. The results indicate that a plasmid expressing E4 from the CMV promoter confers persistent expression from pCF1-CAT, the product of ORF3 alone is sufficient to confer persistent expression, and a single plasmid containing both E4 and CAT is persistent.

5

#### **Example 10: A Plasmid that Expresses E4 ORF3 and CAT**

pCFA-ORF3-CAT was constructed by first inserting a synthetic oligonucleotide containing the rab  $\beta$ -globin polyadenylation signal into the Mfe I site of pCMV(A-N)kan, located 5' of the CMV enhancer promoter. A blunted Taq I fragment containing the Ad2 E4 promoter was then inserted into the Avr II site of pCMV(A-N)kan containing the polyadenylation signal. Ad2 E4 ORF3 was then inserted into the Pme I site. Finally the CAT cDNA was inserted into the Not I site to form pCFA-ORF3-CAT (Figure 23).

15

#### **Example 11: The Requirement for an Additional Component of Adenovirus Other than E4 ORF3 for Optimal Persistence of Expression**

Nude BALB/c mice were instilled with a complex of cationic lipid GL-67 with either pCF1-CAT or pCFA-E4-CAT plus either Ad2/ $\beta$ gal-2, Ad2/ $\beta$ gal-4, or excipient (phosphate buffered saline). CAT assays were performed on lungs harvested at different days post-instillation.

20  
25  
Expression at day 21 from pCFA-E4-CAT + excipient was approximately 20% of day 2 levels, consistent with the results shown in Figure 24. In comparison, expression at day 21 from mice that received pCFA-E4-CAT + Ad2/ $\beta$ gal-2 was greater than 100% of day 2 levels. This suggests that the presence of the adenovirus within the complex increases the persistence of expression.

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Claims

1. A transgene expression system comprising (a) DNA sequences encoding a transcription unit containing a transgene operably linked to expression control sequences, and (b) DNA sequences encoding all or part of the adenovirus E4 region.  
5
2. The transgene expression system of Claim 1 in which the DNA sequences encoding the transcription unit and all or part of the adenovirus E4 region are contained on a plasmid.
3. The transgene expression system of Claim 1 in which the DNA sequences encoding the transcription unit is contained on a plasmid and the DNA sequence encoding all or part of the adenovirus E4 region is contained on an adenoviral vector.  
10
4. The transgene expression system of Claim 1 in which the DNA sequences encoding the transcription unit and all or part of the adenovirus E4 region are contained on an adenoviral vector.
5. The transgene expression system of Claim 1 in which the expression control sequence is selected from the group consisting of the RSV, MUC1 and the CMV promoters.  
15
6. The transgene expression system of Claim 1 in which the expression control sequence is the CMV promoter.
7. The transgene expression system of Claim 1 in which the transgene is cystic fibrosis transmembrane regulator (CFTR).  
20
8. A complex comprising the transgene expression system of Claim 1 and a cationic amphiphile.
9. The complex of Claim 8 in which the cationic amphiphile is GL-67.
10. A method for delivering a transgene to a target cell, comprising combining a transgene expression system and one or more cationic amphiphiles to form a complex, and introducing said complex into a cell.  
25
11. A composition containing the complex of Claim 8 and a physiologically acceptable carrier.

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12. A method of providing CFTR to airway epithelial cells of a cystic fibrosis patient comprising combining one or more cationic amphiphiles and a transgene expression system containing a transgene encoding CFTR to form a complex, and administering an amount of said complex to the airway epithelial cells of said cystic fibrosis patient so as to provide biologically active CFTR protein in order to generate a functional chloride ion channel, under conditions whereby said transgene is expressed and a functional chloride ion channel is produced in the airway epithelial cells of said patient.

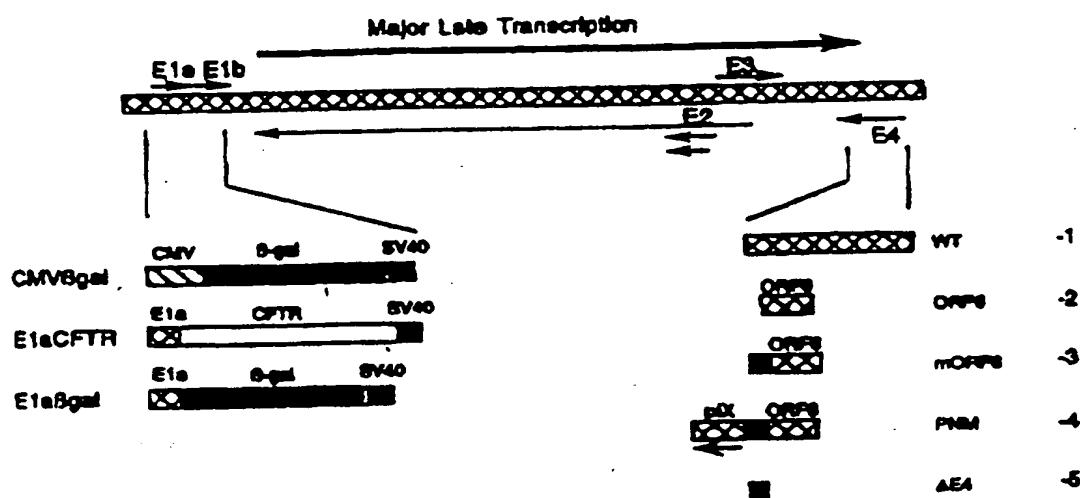
5

13. The transgene expression system of Claim 1 in which the DNA sequences encoding the transcription unit and ORF3 of the adenovirus E4 region are contained on an adenoviral vector.

10

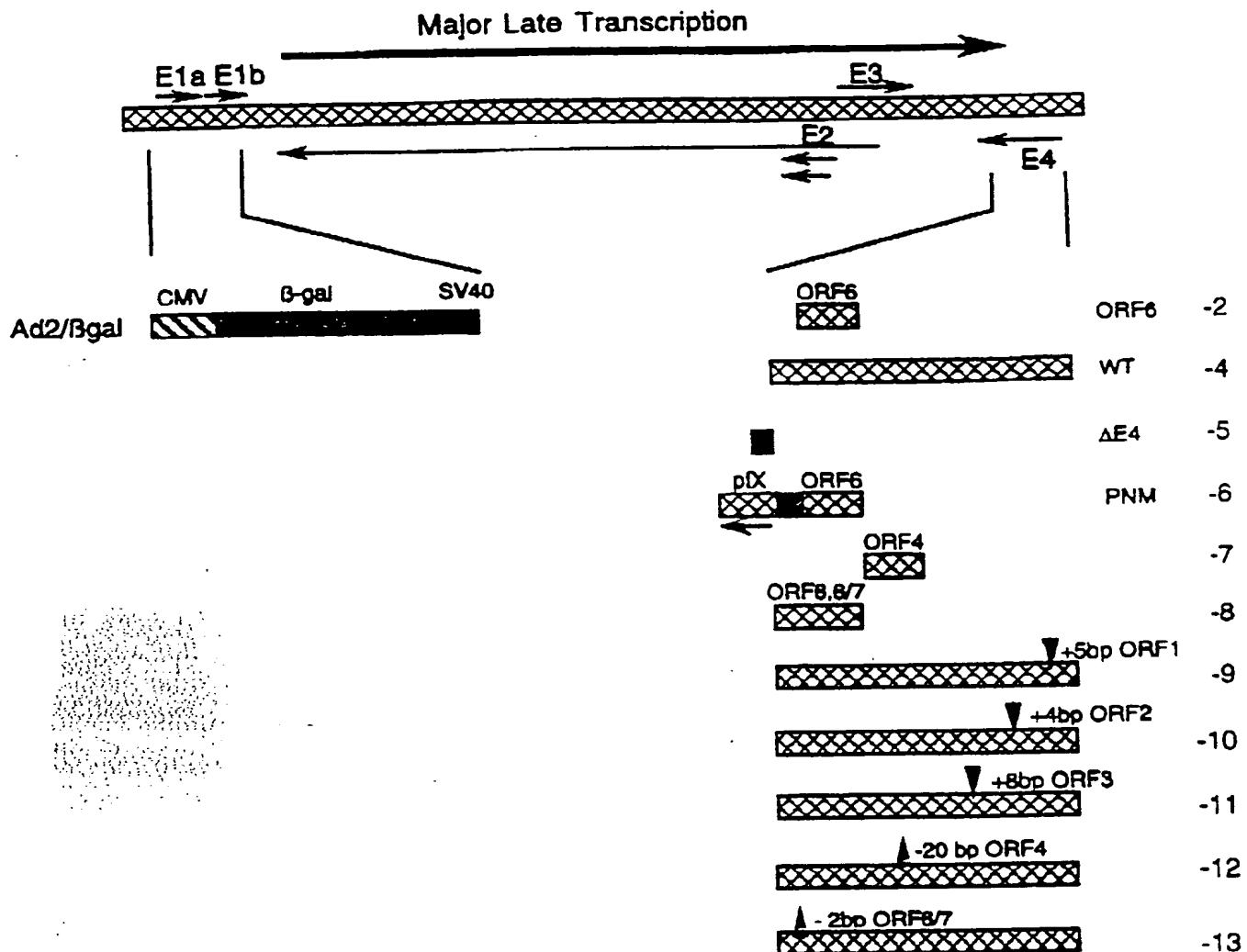
14. The transgene expression system of Claim 1 in which the DNA sequences encoding the transcription unit and ORF3 and ORF4 of the adenovirus E4 region are contained on an adenoviral vector.

15



## FIGURE 1

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## FIGURE 2

## Ad2/CFTR Vector Series

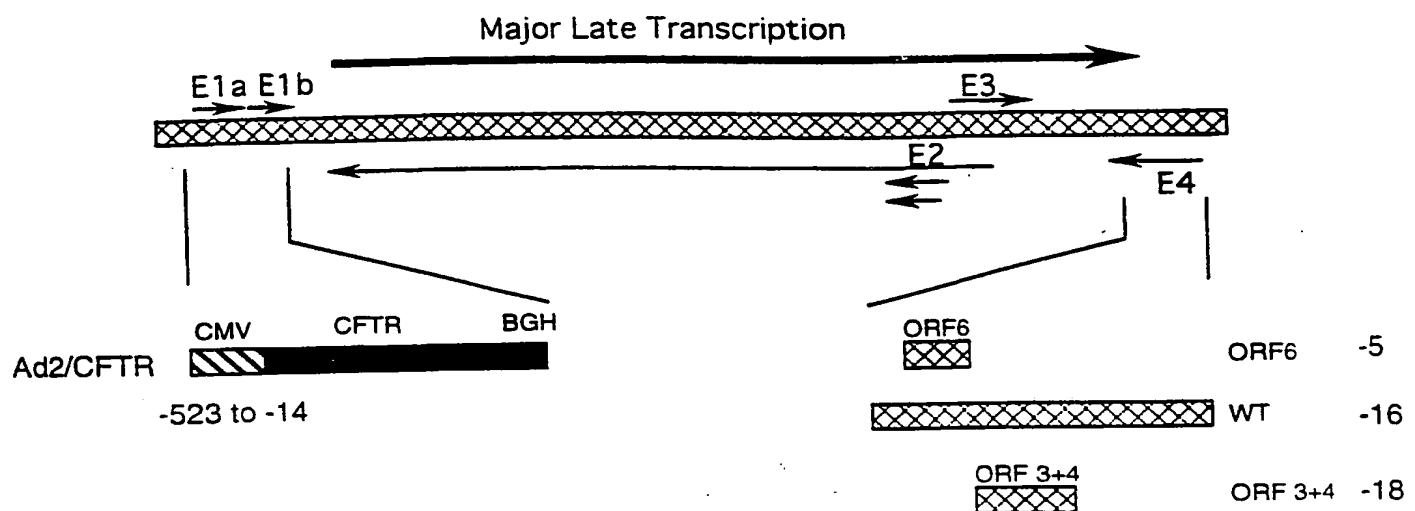


FIGURE 3

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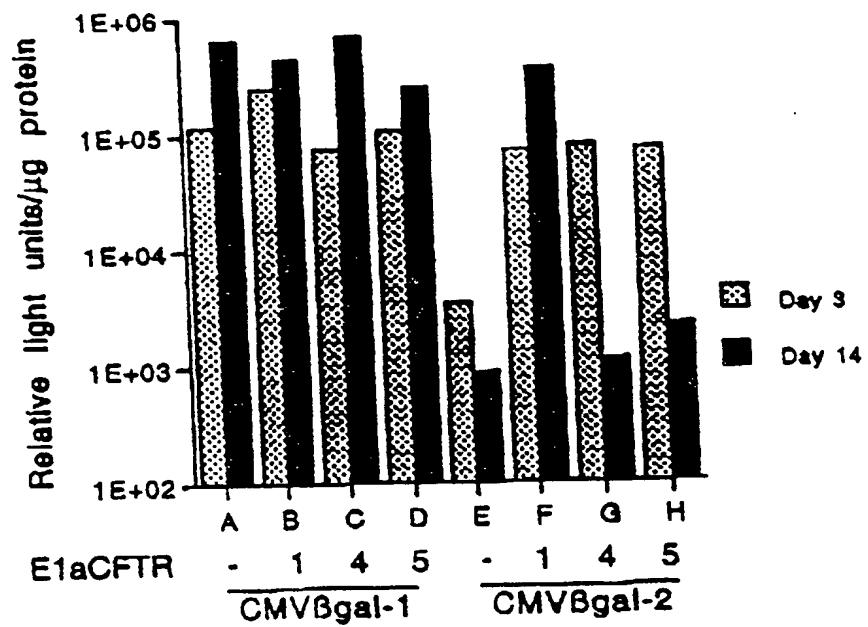


FIGURE 4

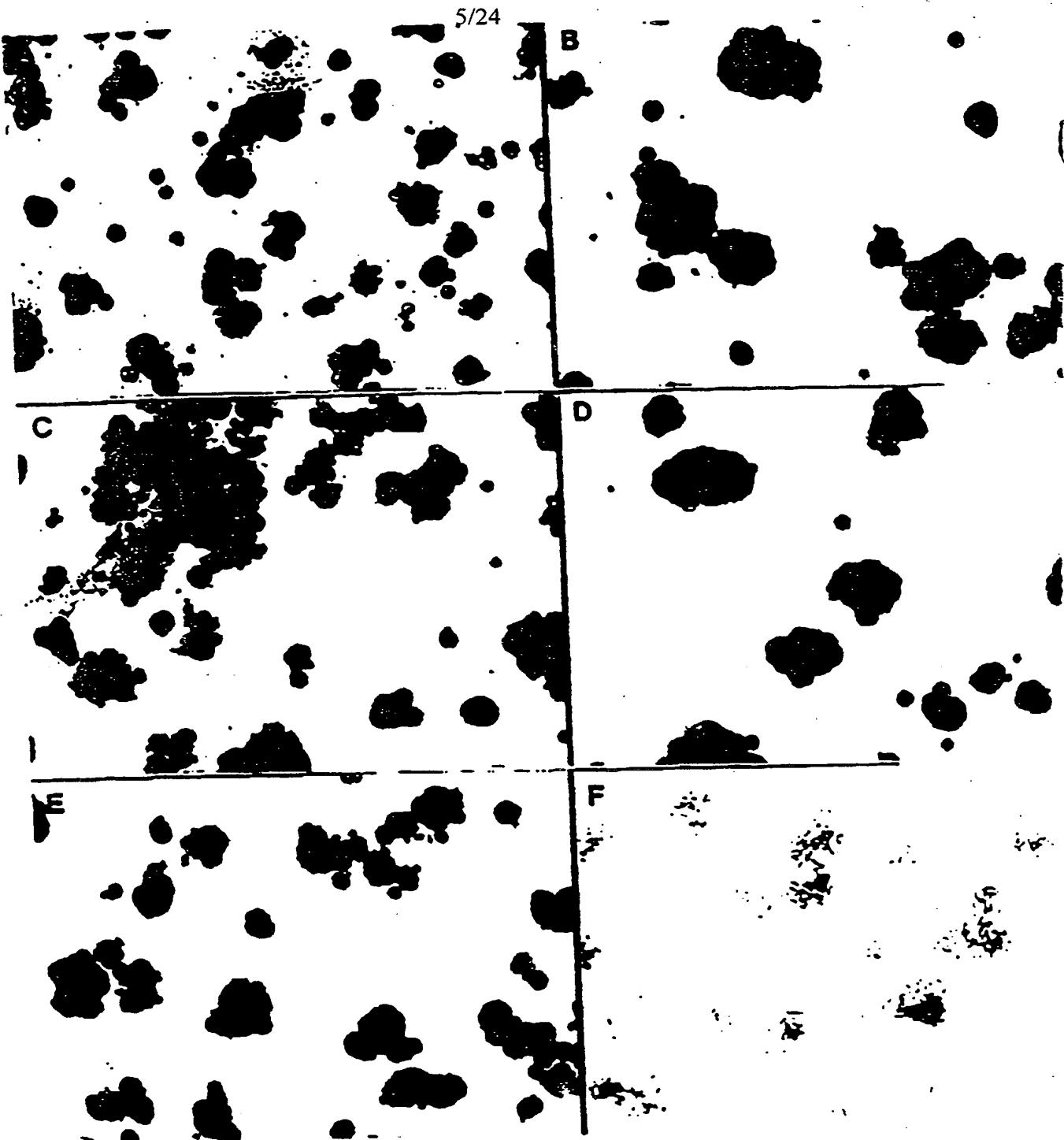


FIGURE 5

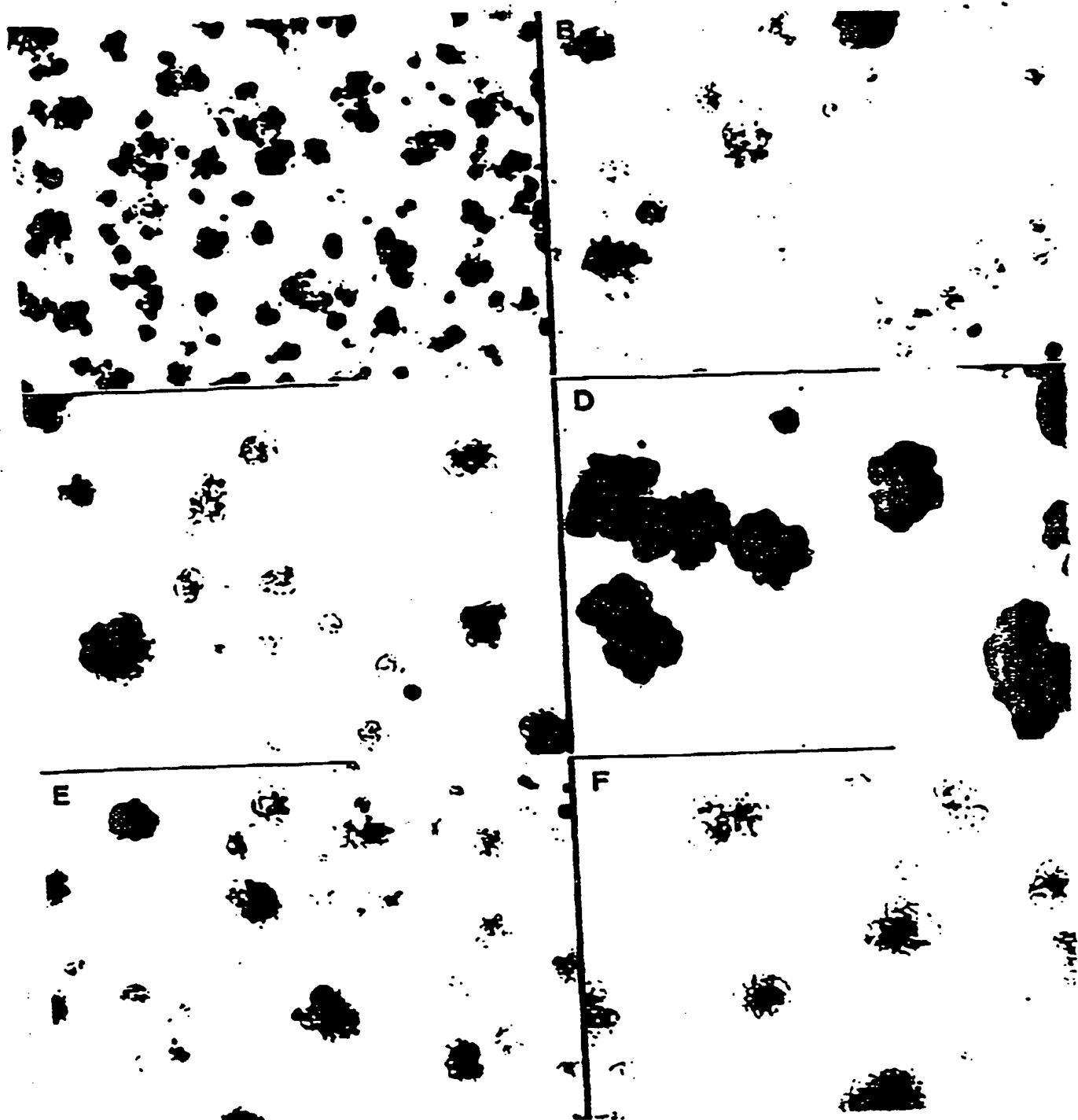


FIGURE 6

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Persistence of  $\beta$ -galactosidase expression in mouse lung

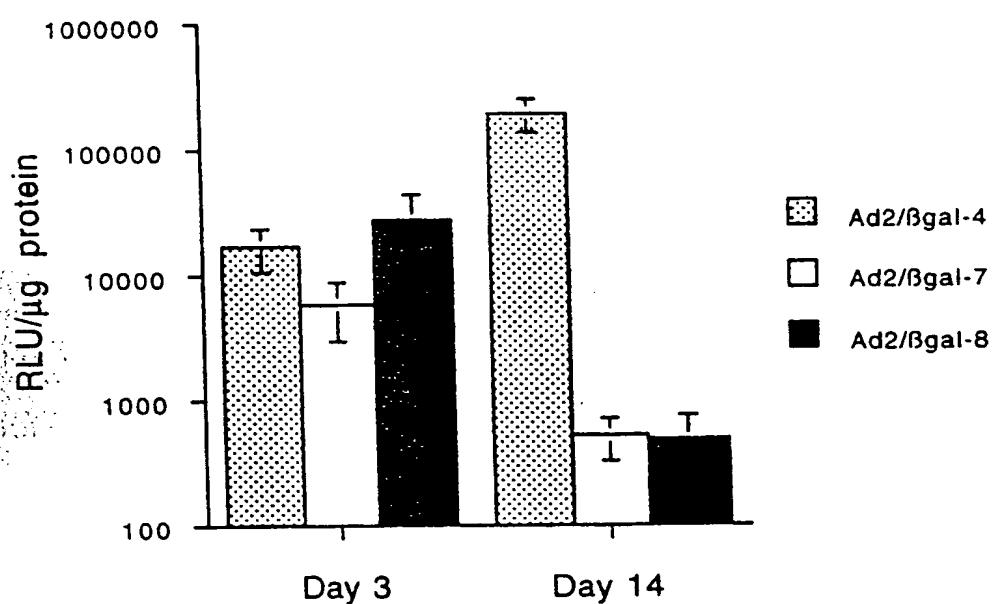


FIGURE 7

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### Effect of E4 ORF3+4 on expression from the CMV promoter

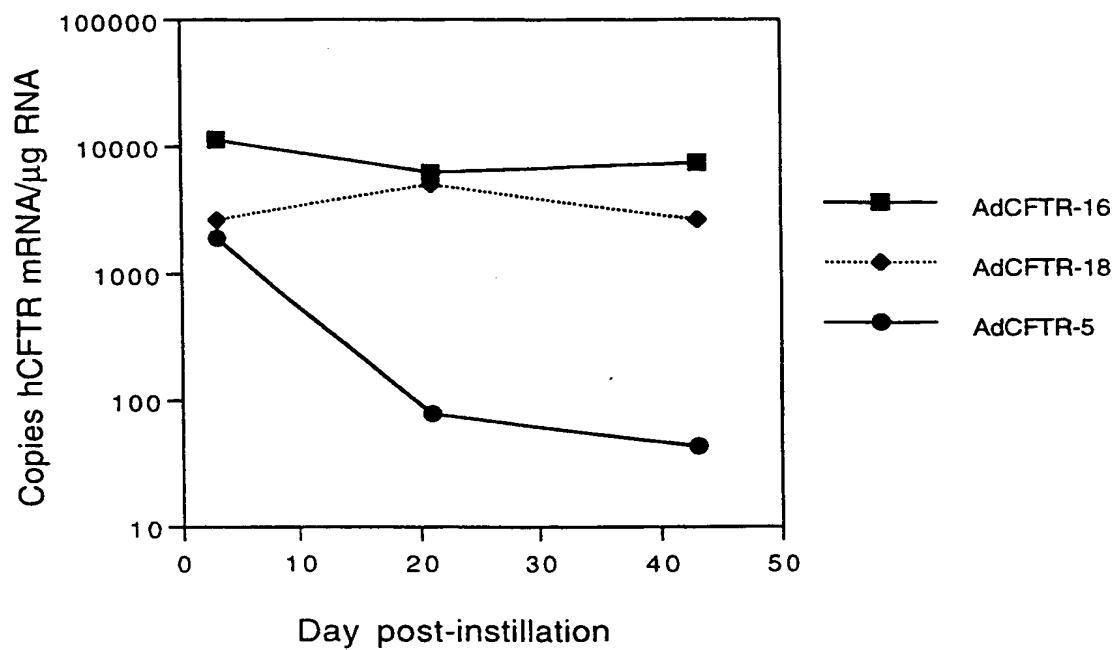
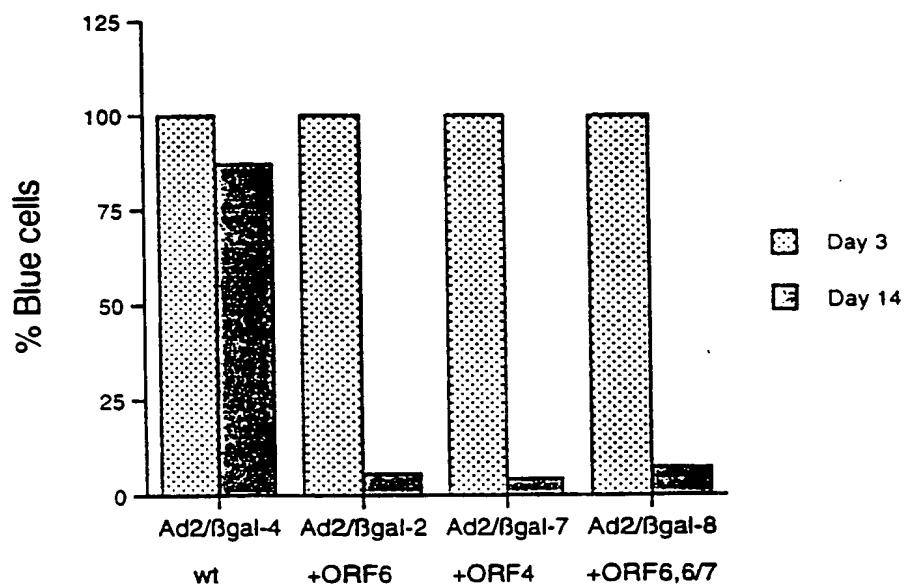


FIGURE 8

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**Persistence of  $\beta$ -galactosidase expression in rat hepatocytes infected with E4 modified viruses**

A.



B.

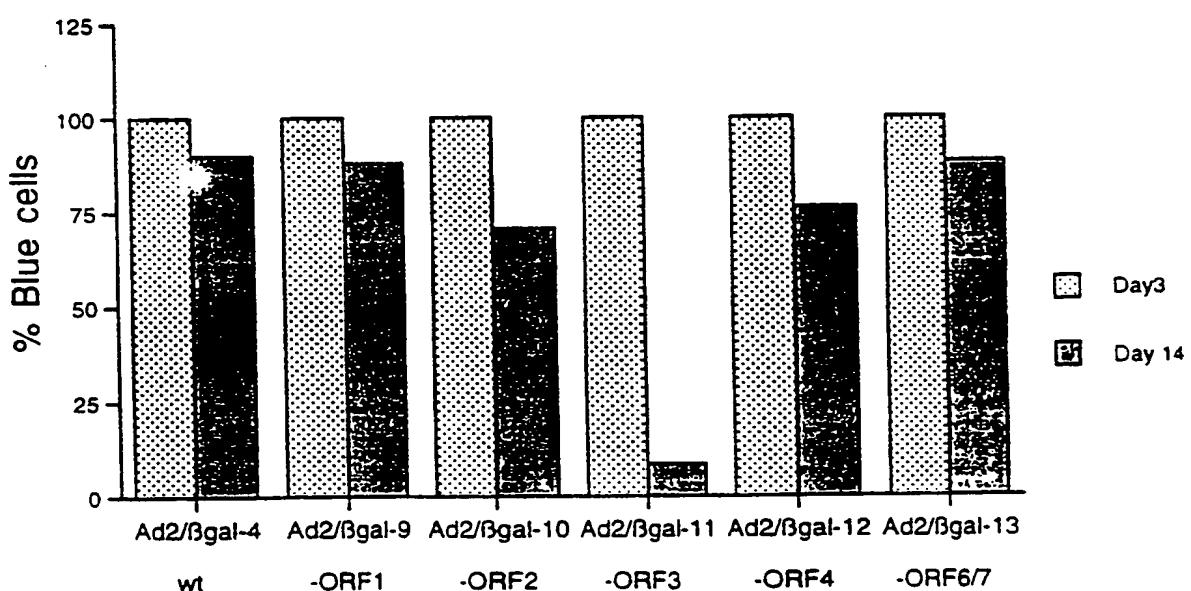


FIGURE 9

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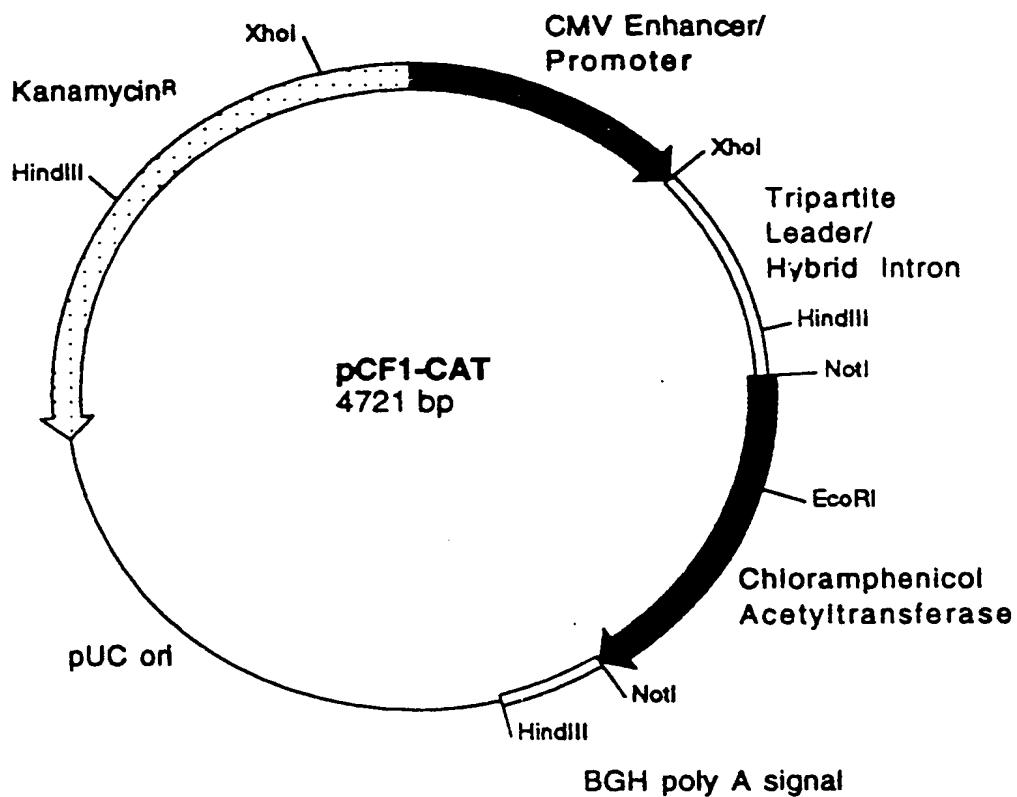
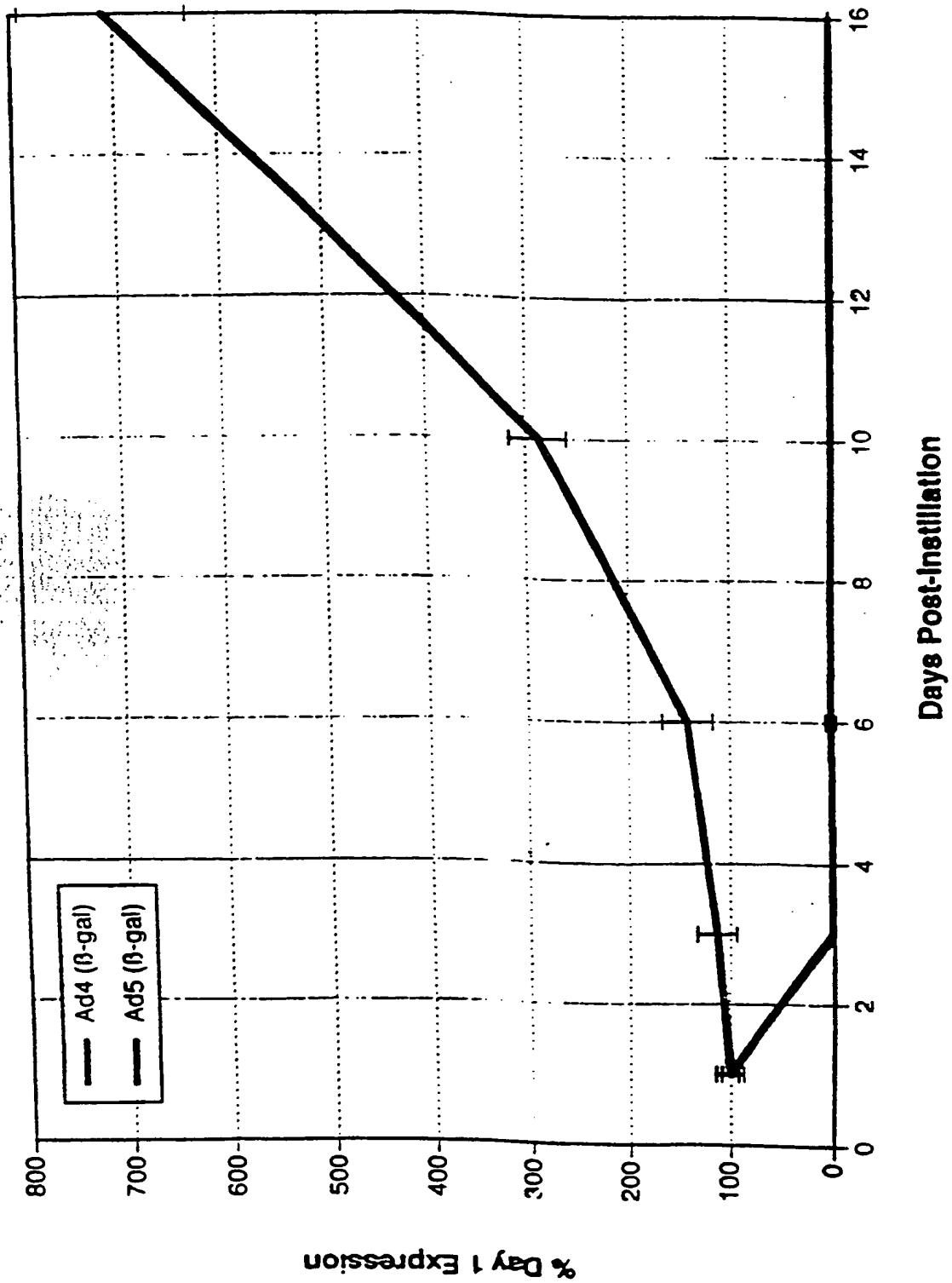


FIGURE 10

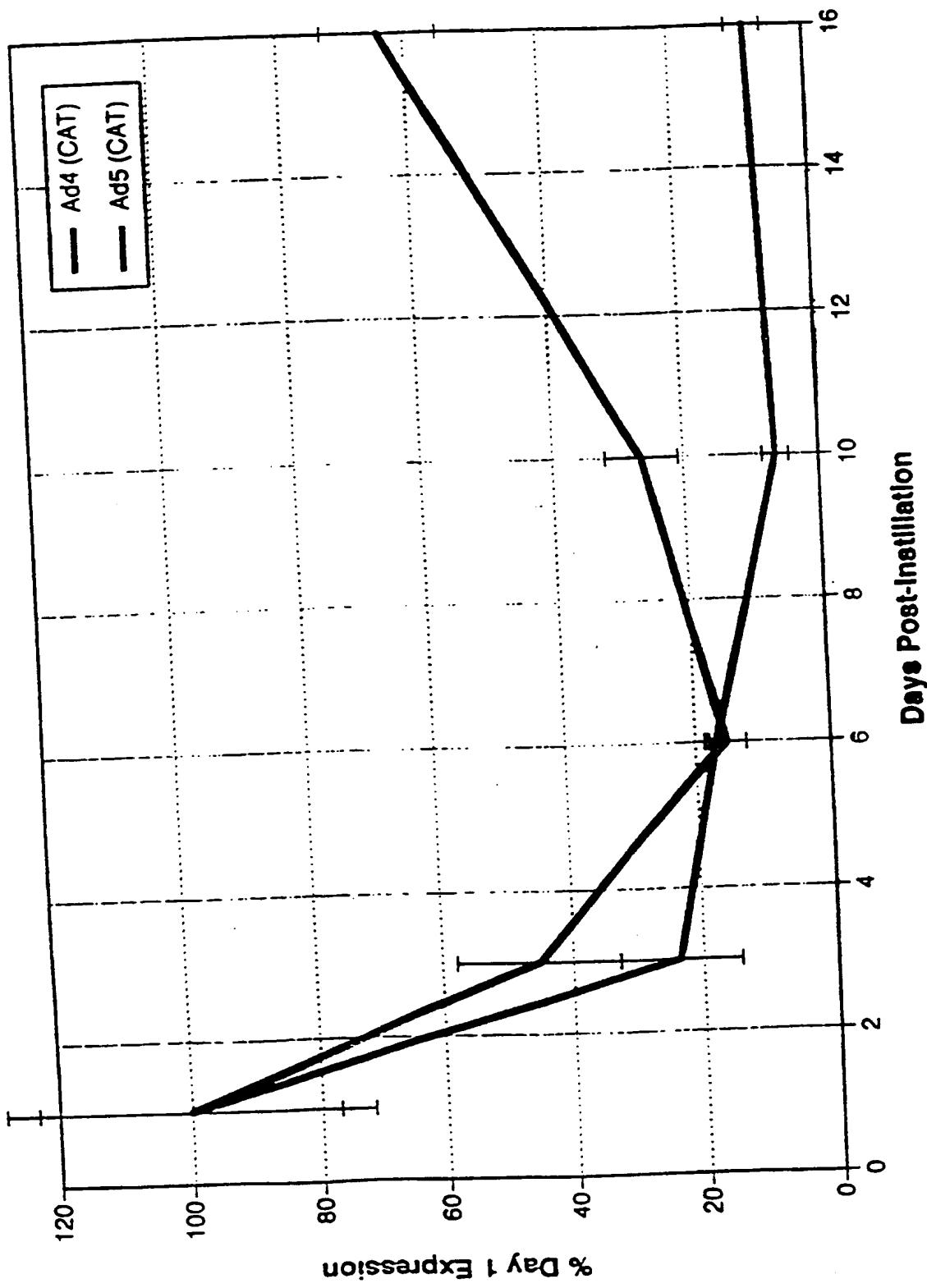
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**Persistence of expression of  $\beta$ -galactosidase from  
Adenovirus ± E4 In Nude Mice**



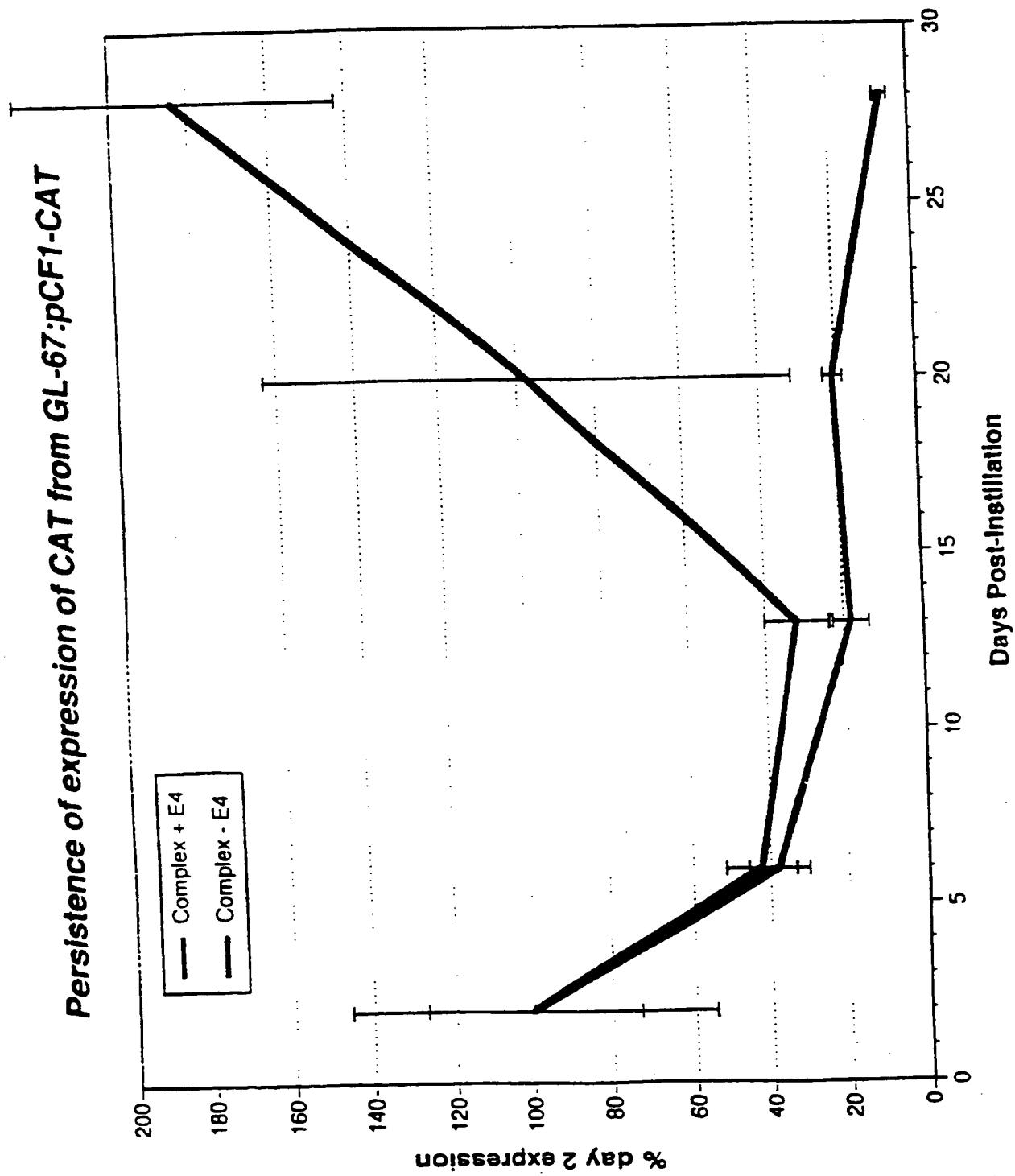
**FIGURE 11**

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## FIGURE 12

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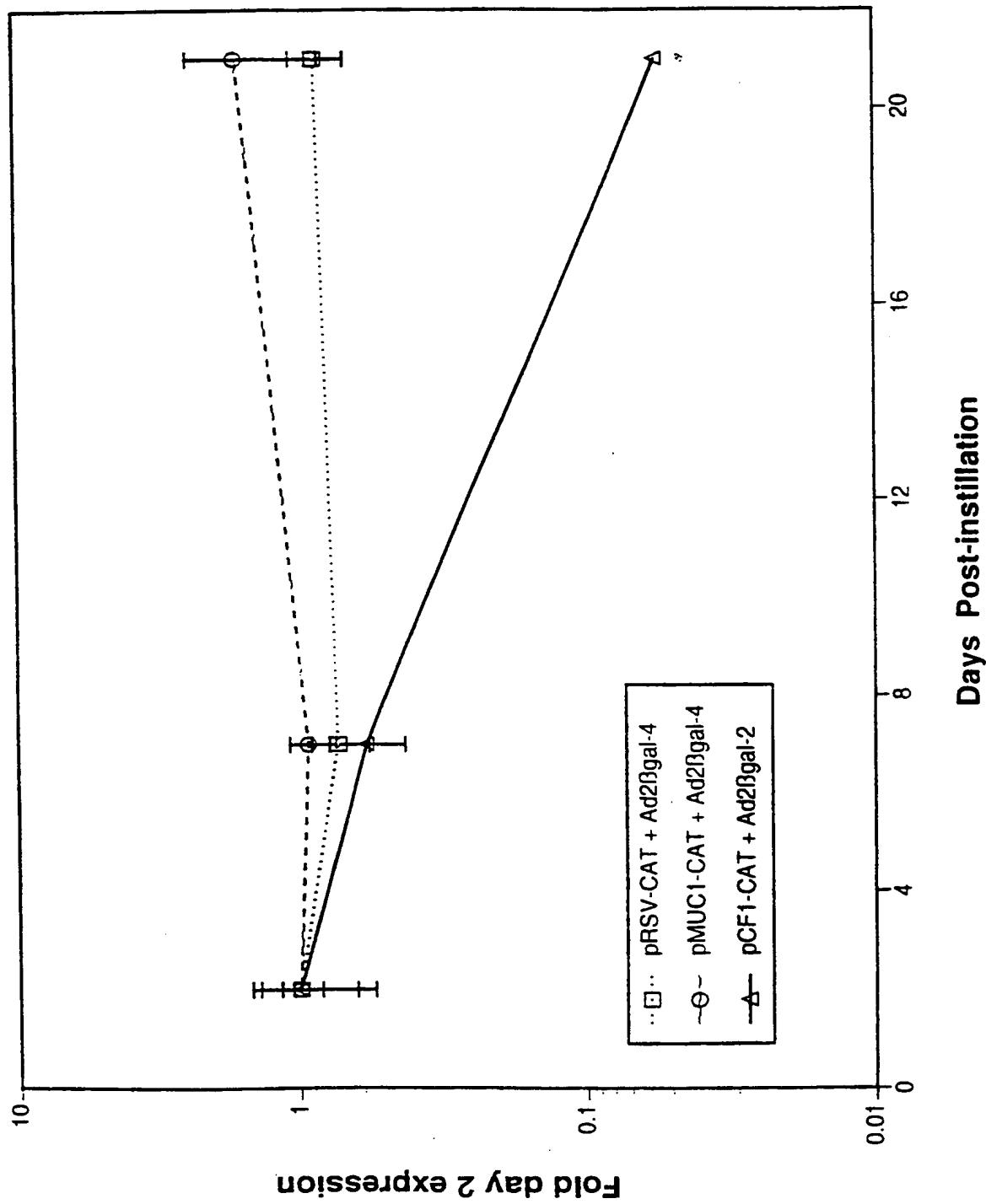


Figure 14

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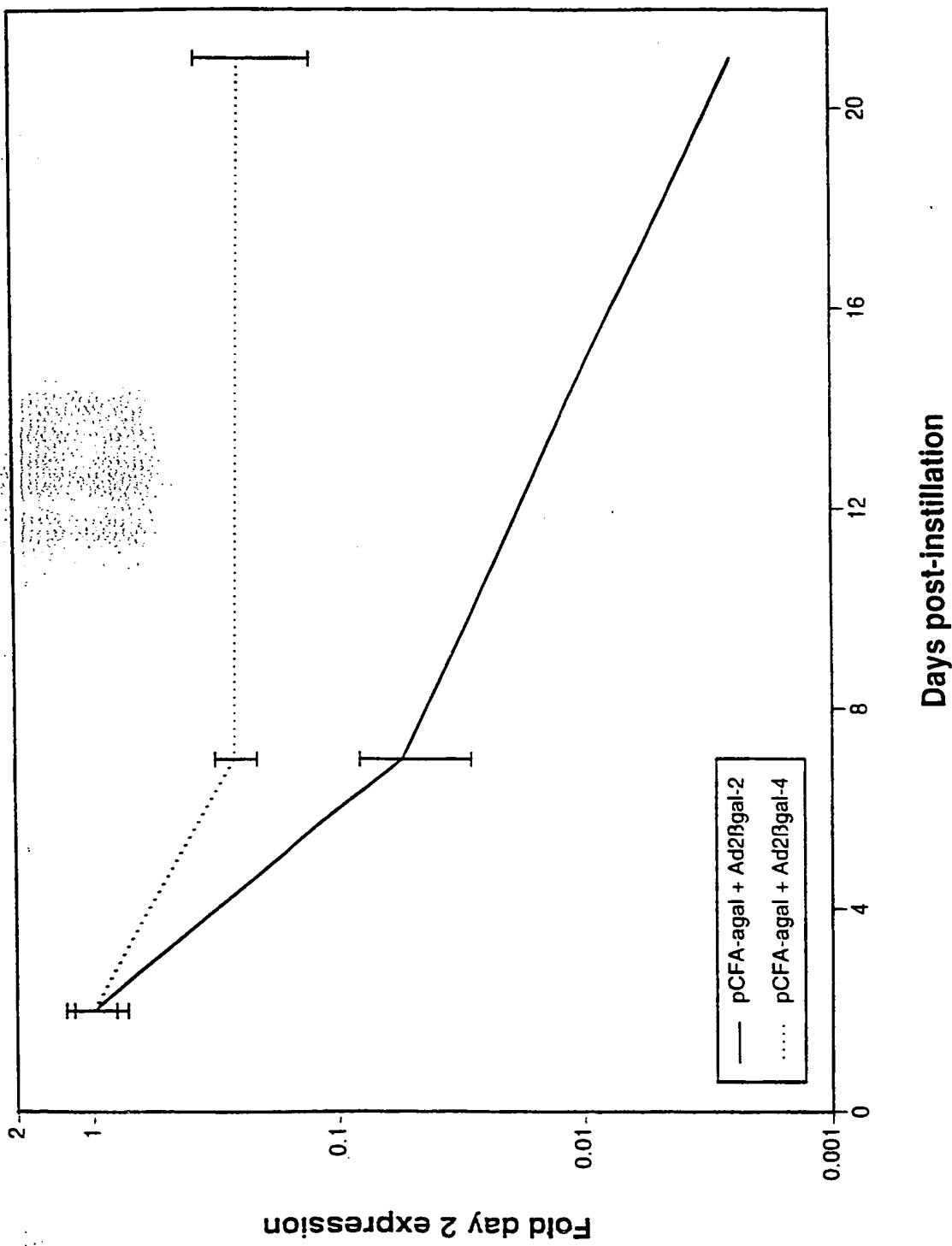


FIGURE 15

Days post-instillation

— pCFA-agal + Ad2 $\beta$ gal-2  
.... pCFA-agal + Ad2 $\beta$ gal-4

Fold day 2 expression

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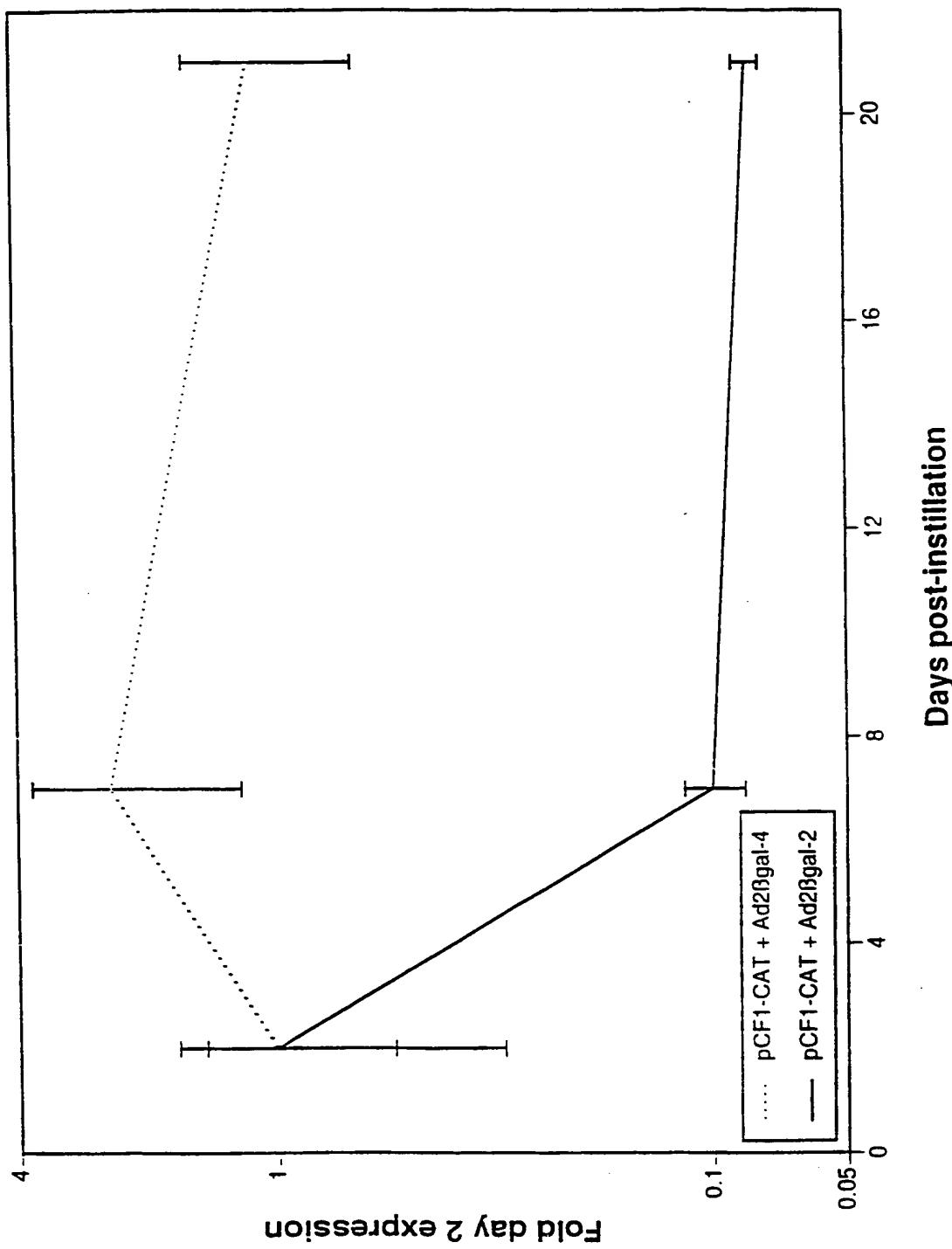


FIGURE 16

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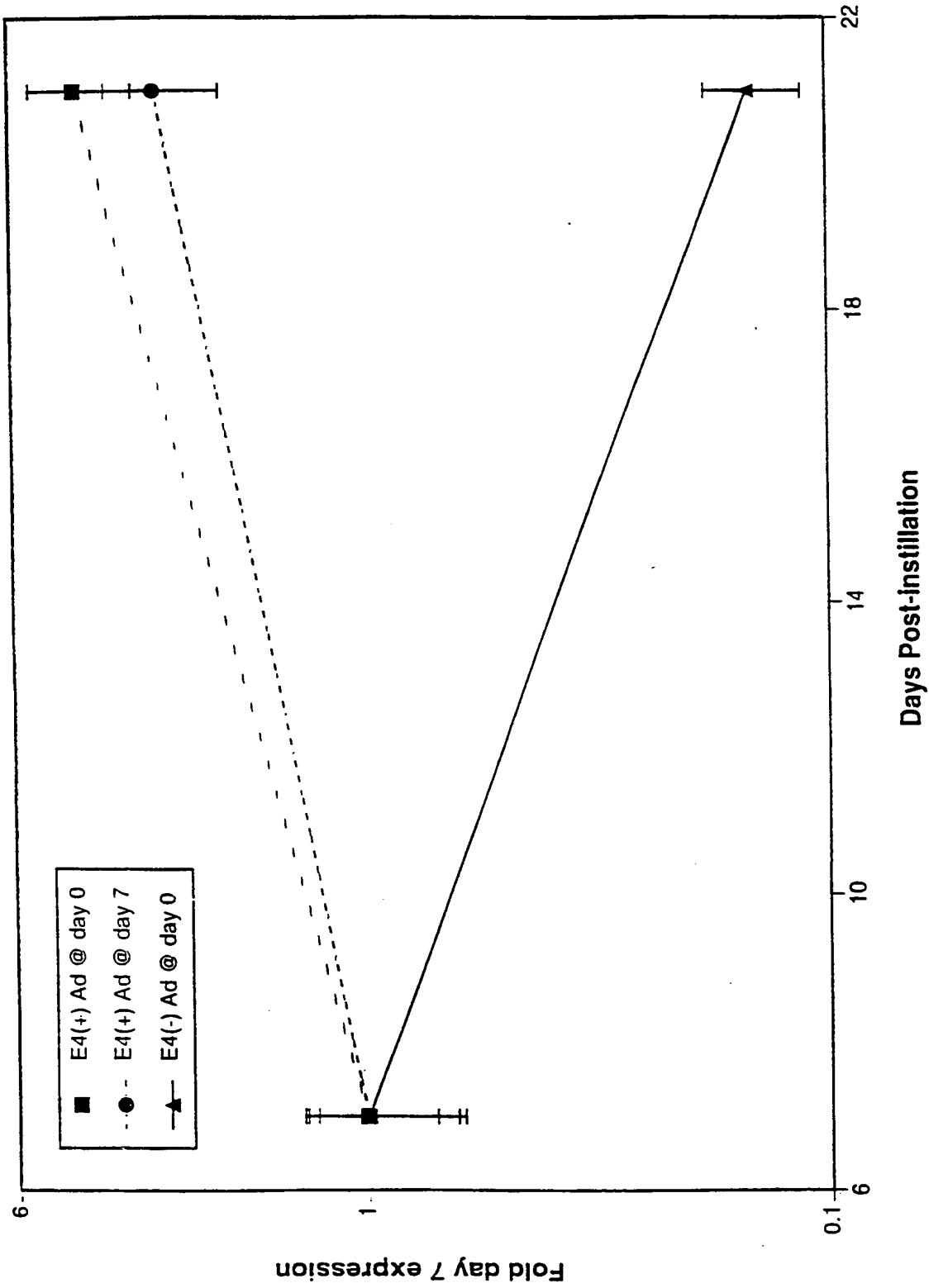


FIGURE 17

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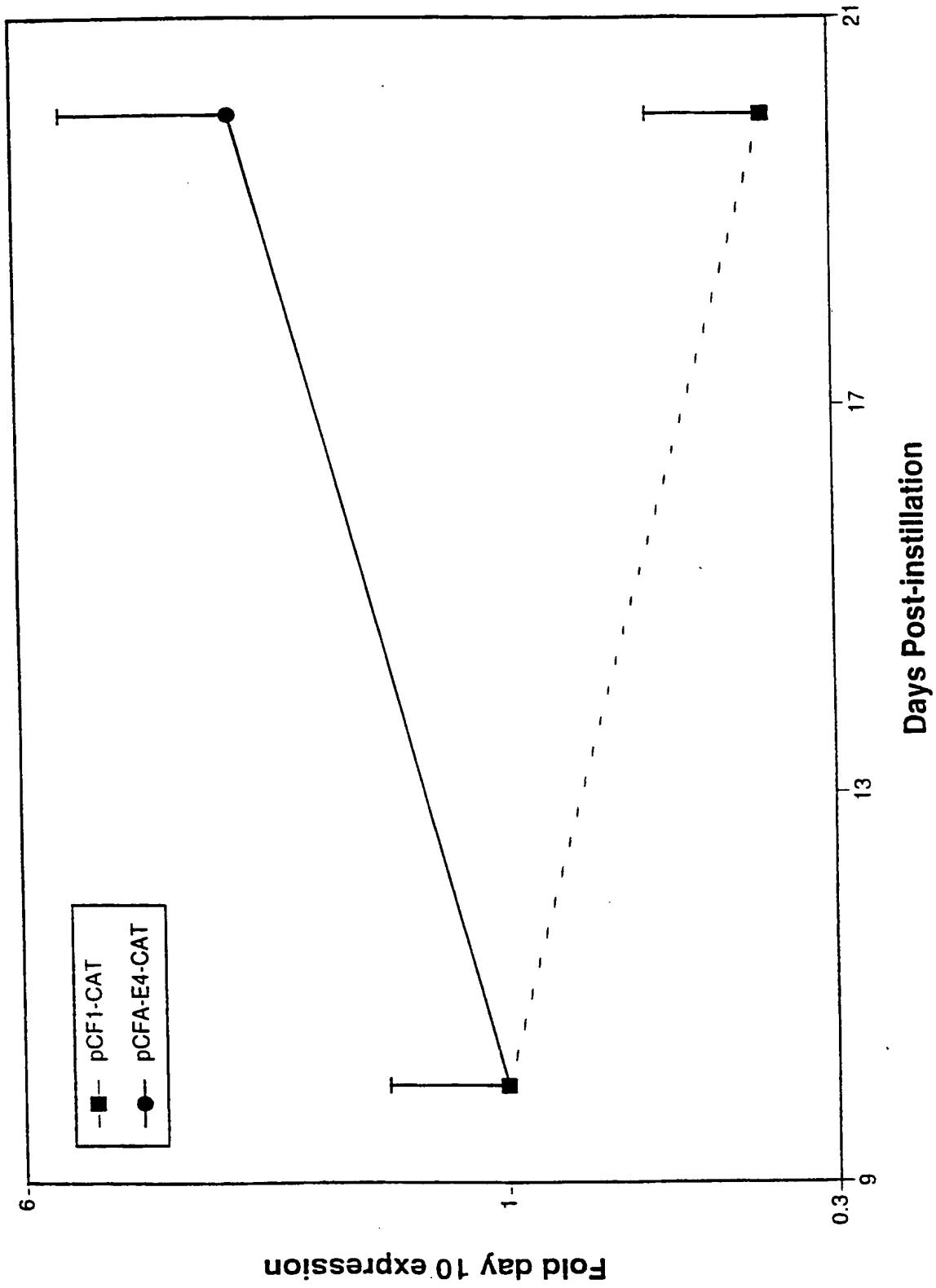


FIGURE 18

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**Expression of CAT from Plasmid vectors with E4+ Adenovirus**

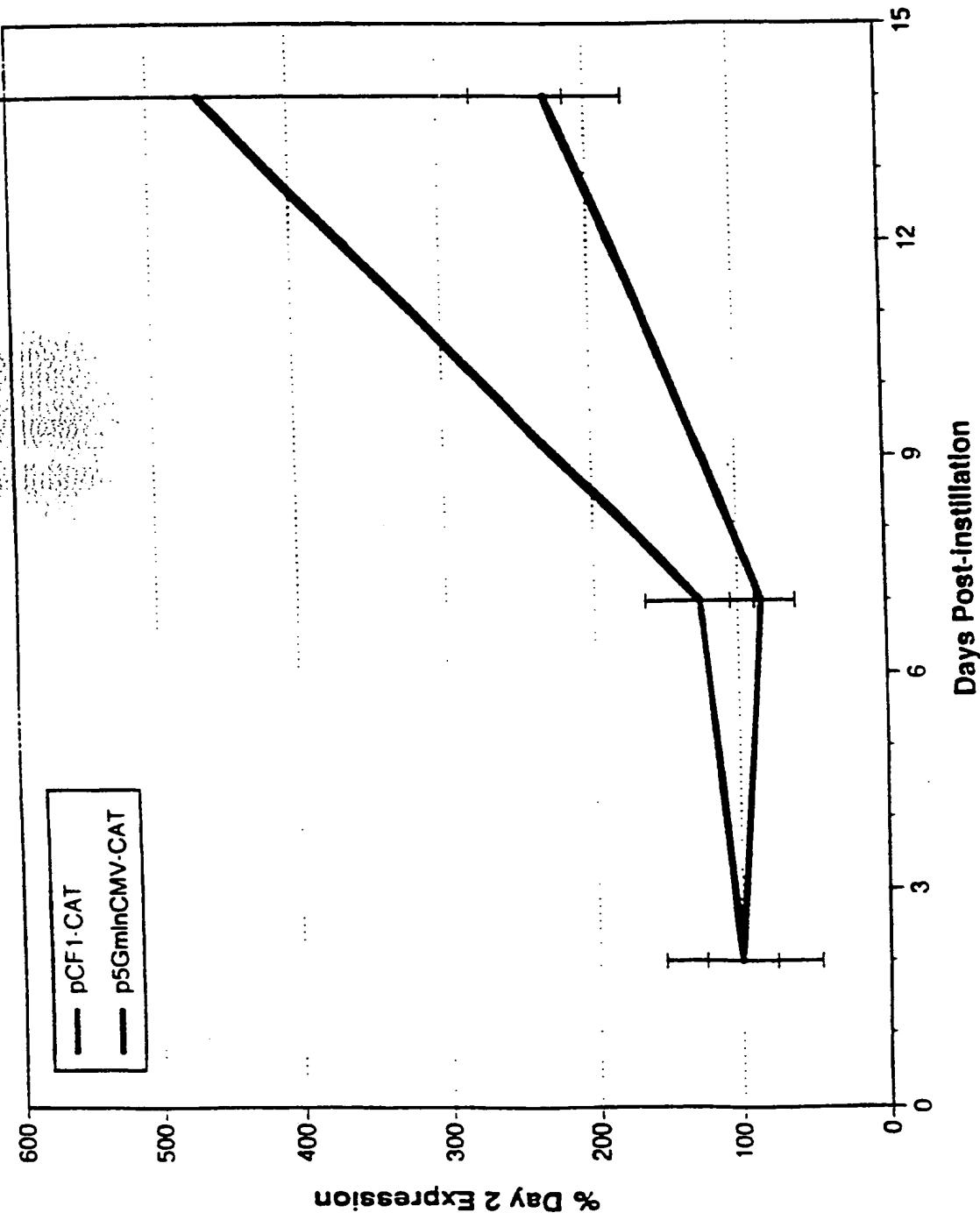


FIGURE 19

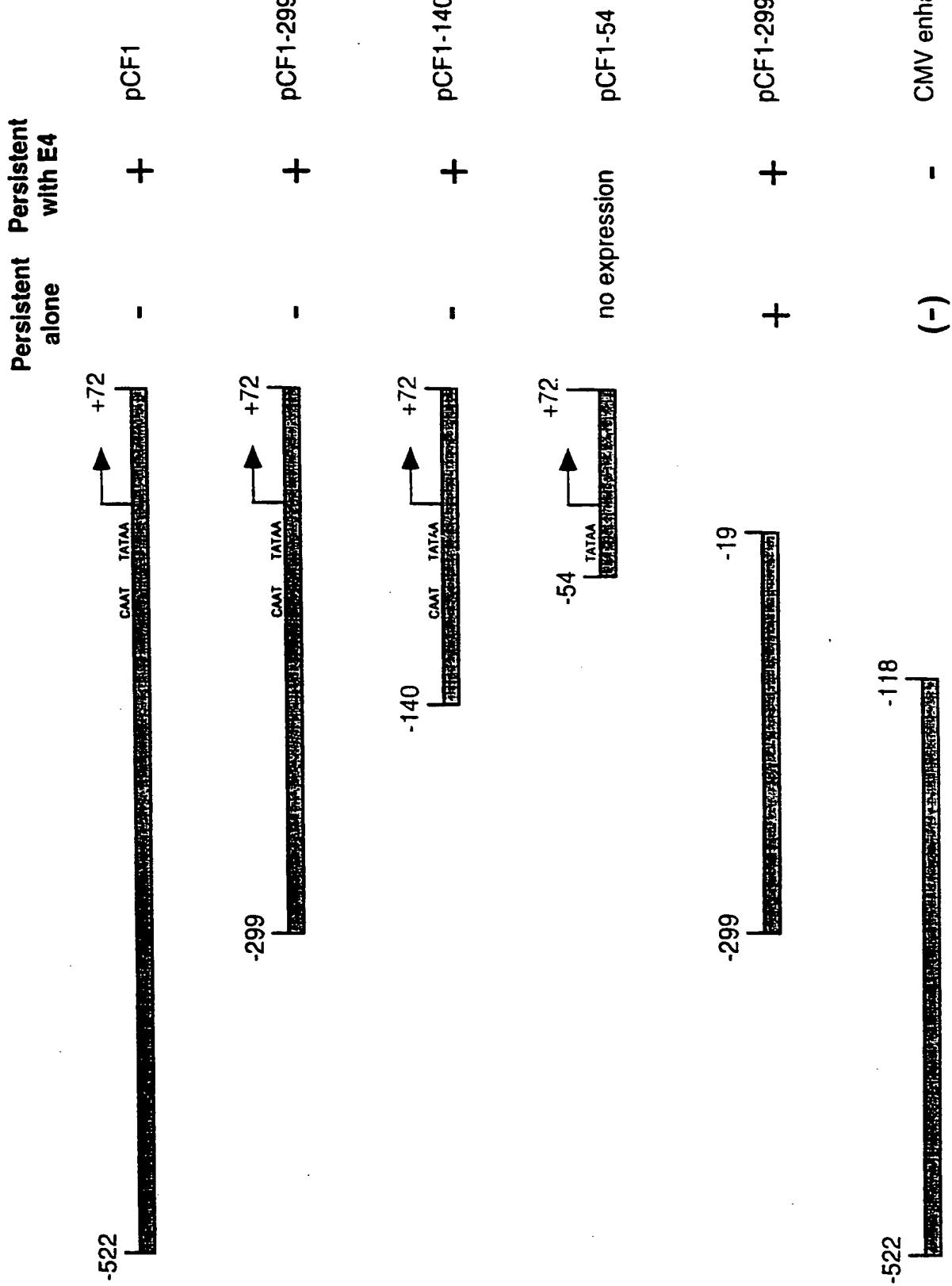


FIGURE 20

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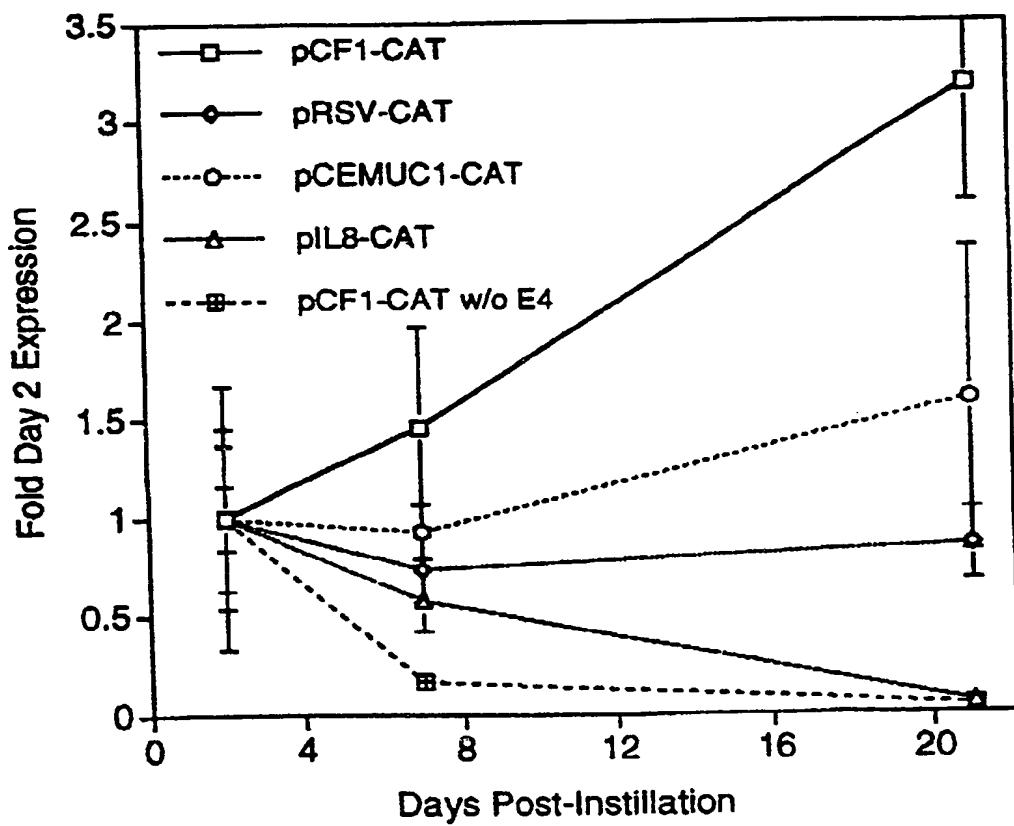


FIGURE 21

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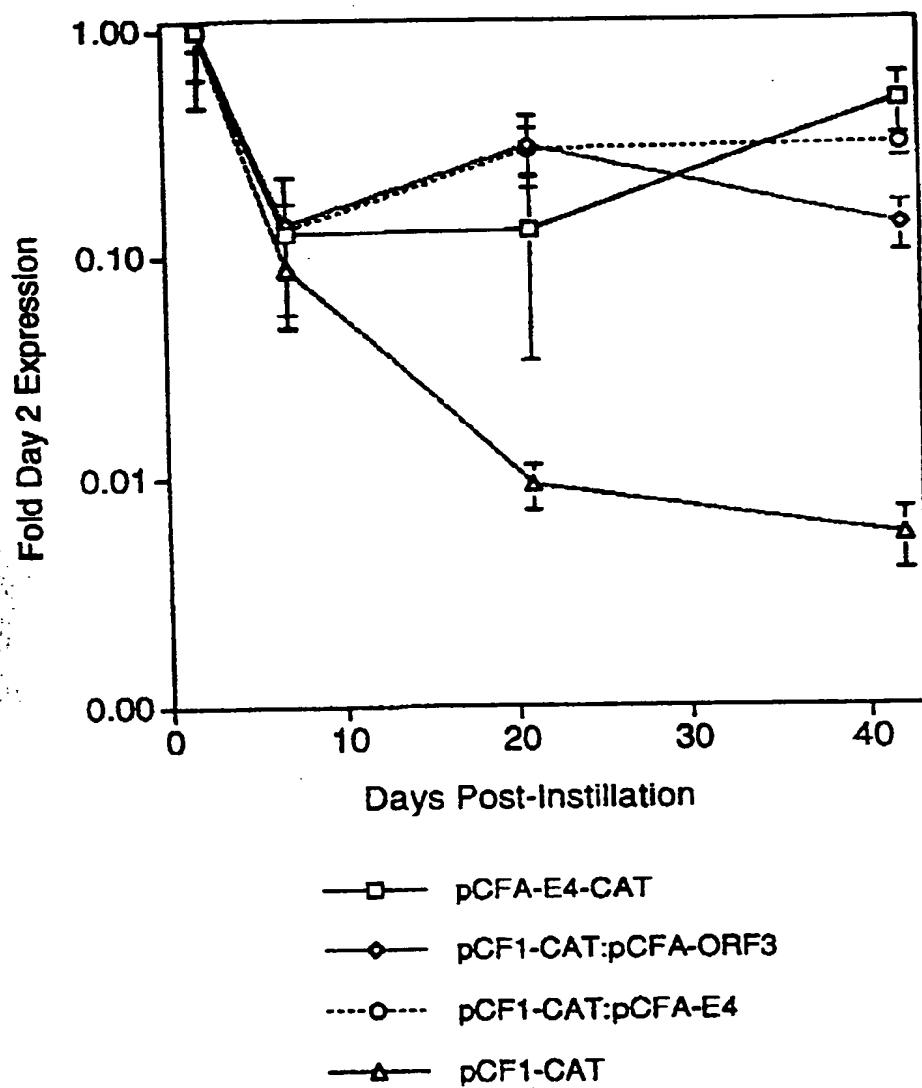


FIGURE 22

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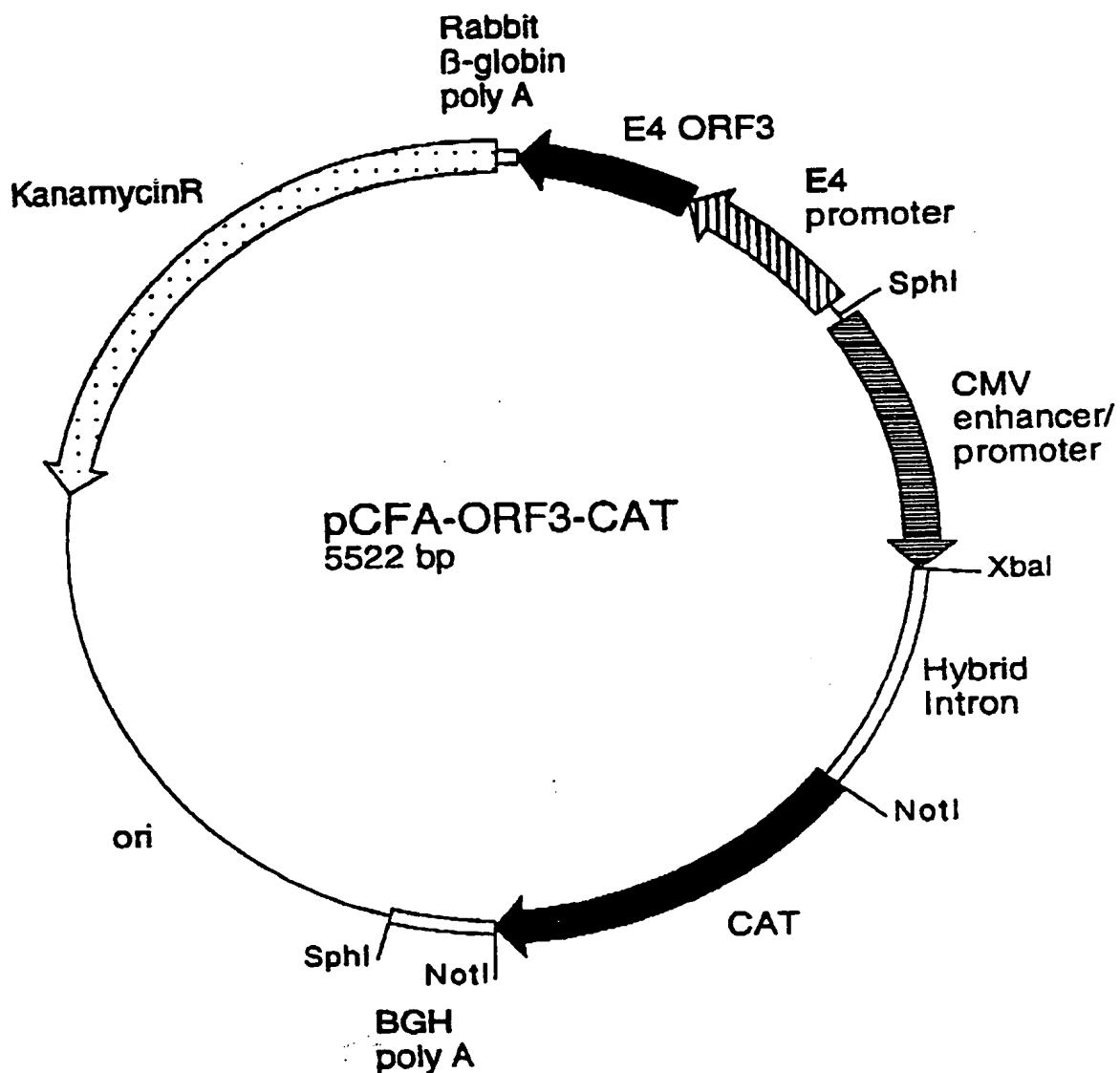


FIGURE 23

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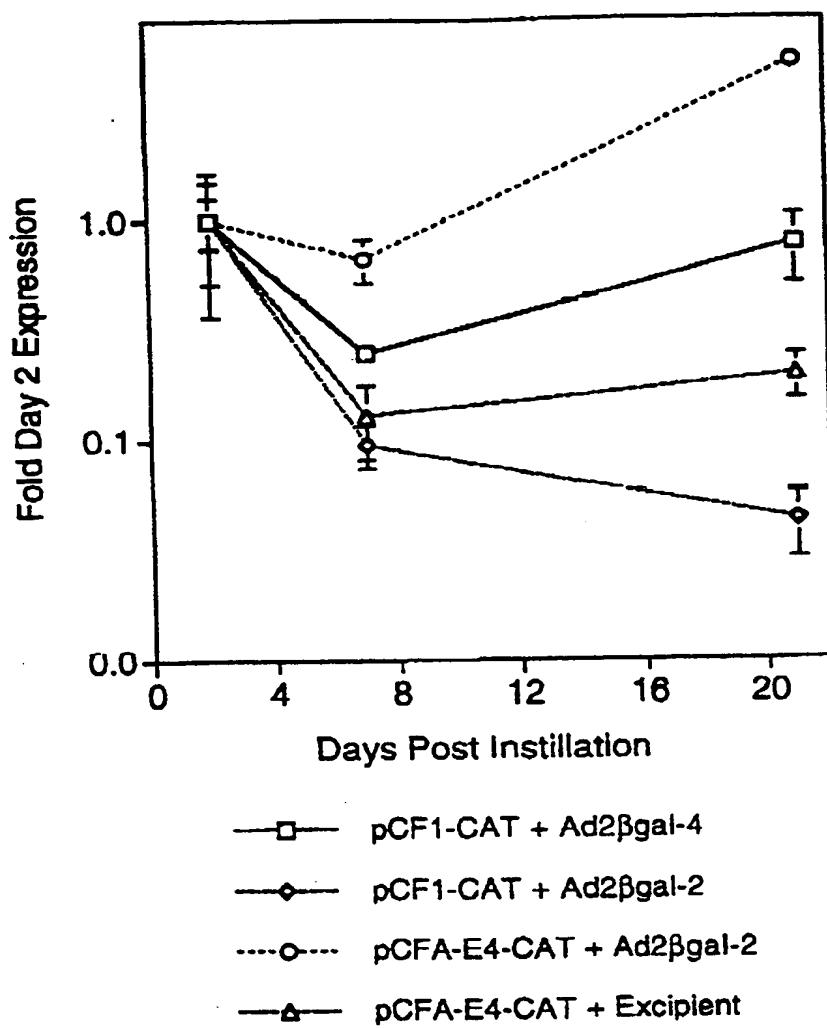


FIGURE 24



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(51) International Patent Classification <sup>6</sup> : <b>C12N 15/86, A61K 48/00</b>		A3	(11) International Publication Number: <b>WO 98/46781</b>
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(21) International Application Number: PCT/US98/07841		(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
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(71) Applicant (for all designated States except US): GENZYME CORPORATION [US/US]; One Mountain Road, Framingham, MA 01701 (US).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): ARMENTANO, Donna [US/US]; 352 Brighton Street, Belmont, MA 02178 (US). MARSHALL, John [GB/US]; 25 Northrop Street, Hopedale, MA 01747 (US). YEW, Nelson [US/US]; 25 Rockdale Hill Circle, West Upton, MA 01568 (US). CHENG, Seng [MY/US]; 50 Wall Street, Wellesley, MA 02181 (US). GREGORY, Richard, J. [US/US]; 2 Wintergreen Lane, Westford, MA 01886 (US).			
(74) Agent: SEIDE, Rochelle, K.; Baker & Botts, LLP, 30 Rockefeller Plaza, New York, NY 10112-0228 (US).			
(54) Title: NOVEL TRANSGENE EXPRESSION SYSTEM FOR INCREASED PERSISTENCE			
(57) Abstract			
<p>The invention is directed to a transgene expression system comprising a transcription unit which contains a transgene operably linked to expression control sequences, preferably the CMV promoter, and which is delivered simultaneously with all or part of the adenovirus E1 genomic region to a cell in order to facilitate persistent expression of the transgene. The components of the transgene expression system can be delivered by vectors including plasmids and/or viruses and may be complexed with cationic amphiphiles to facilitate entry into a cell. The invention is also directed to methods for the production of the transgene expression system. The invention is further directed to compositions that contain the transgene expression system and to methods for the use of such compositions to deliver transgenes encoding biologically active proteins to cells.</p>			

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# INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/US 98/07841

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C12N15/86 A61K48/00

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Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 30534 A (GENZYME CORPORATION) 3 October 1996 * figures; claims * ---	1-8, 13, 14
X	WO 96 13596 A (RHONE POULENC ROHRER) 9 May 1996 * claims 1-11 * ---	1-4, 13, 14
X	WO 94 28938 A (THE REGENTS OF THE UNIVERSITY OF MICHIGAN) 22 December 1994 * claims 1,9,18; pages 3,4 * ---	1-8, 10-14

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BRIDGE, E. ET AL.: "Adenovirus early region 4 and viral DNA synthesis" VIROLOGY, vol. 193, 1993, pages 794-801, XP002051623 * page 799, right-hand column, first paragraph * ---	1-4, 13, 14
A	WO 96 18372 A (GENZYME CORPORATION) 20 June 1996 cited in the application * p. 1-17 * -----	8-12

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 98/07841

### Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
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4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

#### Remark on Protest

The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-7,13,14

A transgene expression system

2. Claims: 8,9,11

A complex comprising a transgene expression system and a cationic amphiphile

3. Claims: 10,12

A method for delivering a transgene to a cell

## INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l. application No

PCT/US 98/07841

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
WO 9630534	A 03-10-1996	US 5707618 A			13-01-1998
		AU 5318496 A			16-10-1996
		EP 0843731 A			27-05-1998
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		EP 0710123 A			08-05-1996
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WO 9618372	A 20-06-1996	US 5650096 A			22-07-1997
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